



# miR-25-3p ameliorates SAE by targeting the TLR4/NLRP3 axis

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## Abstract

Sepsis-associated encephalopathy (SAE) is a severe complication of sepsis. It has been reported that miR-25-3p is closely related to the development of sepsis. However, the detailed mechanism of miR-25-3p in SAE requires further investigation. Caecum ligation and puncture (CLP) was performed to induce SAE in vivo. LPS stimulation was applied to mimic the in vitro inflammatory model. The expression levels of TLR4 and NLRP3 in the cerebral cortex were evaluated by immunofluorescence. The gene and protein expression levels were determined by qRT-PCR and a western blot analysis. ELISA was used to detect the levels of inflammatory cytokines. The interaction between miR-25-3p and TLR4 was validated by a dual luciferase reporter assay. TLR4 and NLRP3 were highly expressed in the cerebral cortex of SAE mice, while miR-25-3p was expressed at low levels. Activation of the inflammasome, increased release of cytokines and microglial activation were also observed in the SAE mouse model. The overexpression of miR-25-3p inhibited the expression of LPS-induced cytokines and microglial activation. Furthermore, miR-25-3p inhibited TLR4 expression by directly targeting TLR4. The anti-inflammatory effect of miR-25-3p in LPS-induced CHME5 was reversed by TLR4 overexpression. miR-25-3p overexpression attenuated the activation of microglia in SAE by inhibiting the NLRP3/IL-1 $\beta$ /IL-18 axis by directly targeting TLR4, suggesting that miR-25-3p may be a potential target for SAE diagnosis and treatment.

**Keywords** Sepsis-associated encephalopathy · miR-25-3p · TLR4 · Microglia · NLRP3

## Introduction

Sepsis refers to life-threatening organ dysfunction caused by a host's dysfunctional response to infection (Huang et al. 2019). Sepsis is a life-threatening and multifactorial disease with a continuously increasing incidence (Chung et al. 2020). Currently, sepsis and septic shock with subsequent multiorgan failure are the leading causes of death in adult

intensive care units (ICUs) (Berg and Gerlach 2018). Sepsis-associated encephalopathy (SAE) is one of the most common complications during the acute phase and later stages after surviving sepsis and is defined by diffuse cerebral dysfunction due to a dysregulated host response and absence of a direct central nervous system (CNS) infection (Chung et al. 2020). The clinical symptoms of SAE include cognitive dysfunction, delirium, and deep coma, which seriously affect the quality of life of patients. In addition, many aetiologies contribute to the occurrence of SAE, including blood–brain barrier (BBB) compromise, inflammatory cytokines, and brain injury (Gofton and Young 2012). Thus, further investigation of the mechanism by which microglial activation participates in SAE may provide a better understanding of the pathology of SAE.

Microglia constitute a type of glial cell. Microglia are the first and most effective immune defence line in the CNS. Twenty percent of the glial cells in the brain are microglia (Ma et al. 2017). Numerous previous studies have illustrated that microglial activation plays a critical role in the pathogenesis of neurodegenerative diseases (Hansen et al. 2018). However, the excessive activation of microglia results in

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severe neurotoxicity (Wolf et al. 2017). Microglia can be categorized into the following two subtypes with opposite functions: the proinflammatory M1 type and the anti-inflammatory M2 type (Tang and Le 2016). Microglia can be stimulated by LPS or IFN- $\gamma$  to an M1 phenotype, resulting in the expression of proinflammatory cytokines, or by IL-4/IL-13 to an M2 phenotype, resulting in the resolution of inflammation and tissue repair (Orihuela et al. 2016). Many studies have revealed that the inhibition of the excessive activation of microglia can ameliorate SAE (Mazeraud et al. 2020). However, the detailed molecular mechanism remains unclear.

MicroRNAs (miRNAs) constitute a subclass of noncoding RNAs ~22 nt in length that participate in regulating many biological processes (Bartel 2004). MiRNAs are epigenetic regulators of mRNA that downregulate their expression by binding specific sequences of the 3'-UTR of mRNA (Moore et al. 2013). As widely reported, the progression of SAE is closely associated with the abnormal regulation of miRNAs (Osca-Verdegel et al. 2021). For example, plasma miR-370-3p was identified as a biomarker of SAE by using a transcriptomic profiling analysis (Visitchanakun et al. 2020). In addition, Sun et al. revealed that miR-155-5p expression was significantly reduced in microglia and cortical tissue following the induction of endotoxin tolerance and that miR-155-5p inhibition contributed to the development of endotoxin tolerance (Sun et al. 2018). It was reported that miR-25 was a biomarker of sepsis (Benz et al. 2016). A functional study revealed that miR-25-3p attenuated sepsis-induced cardiomyocyte apoptosis *in vivo* and *in vitro* (Yao et al. 2018). A previous study showed that miR-25-3p could directly target PTEN to inhibit apoptotic signalling. Moreover, miR-25-3p was reported to target HMGB1 to prohibit the activation of NF- $\kappa$ B signalling and suppress the expression of proinflammatory cytokines in sepsis (Zhu et al. 2018). However, whether miR-25-3p is involved in microglial activation in SAE remains unknown.

The NLR family pyrin domain containing 3 (NLRP3) inflammasome is a type of intracellular polyprotein complex that is mainly composed of the NOD-like receptor family member NLRP3, adaptor protein ASC, and precursor caspase-1 (pro-caspase-1). Inflammasome activation promotes the secretion of interleukin (IL)-1 $\beta$  and IL-18 to increase the inflammatory response (Heneka et al. 2013). NLRP3/IL-1 $\beta$ /IL-18 axis activation was previously reported to aggravate inflammatory injury in sepsis (Hu et al. 2021; Sui et al. 2016). In SAE, a previous study indicated that the inhibition of the NLRP3/IL-1 $\beta$ /IL-18 axis alleviated cognitive dysfunction by attenuating microglial activation (Sui et al. 2016). As a major receptor responsible for the inflammatory response upon sepsis, Toll-like receptor 4 (TLR4) and relevant signalling are positive upstream regulators of NLRP3 inflammasome activation (Tan et al. 2017). Based on a bioinformatics analysis, we revealed that the 3'-UTR of TLR4

possessed a potential binding site for miR-25-3p. However, the regulatory mechanism between TLR4 and miR-25-3p in SAE remains unclear.

In the present study, we investigated the protective effect of miR-25-3p in SAE. The results showed that miR-25-3p repressed microglial activation in SAE by prohibiting the NLRP3/IL-1 $\beta$ /IL-18 axis by directly targeting TLR4. Our study might provide a novel molecular target in SAE.

## Materials and methods

### Mouse SAE model

Male C57BL/6 mice (6–8 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). In the present study, caecal ligation and puncture (CLP) was employed to induce SAE in C57BL/6 mice. The mice were randomly divided into the following 4 groups: sham, CLP 12 h, CLP 24 h, and CLP 48 h. All groups were fasted for 12 h before the operation. Pentobarbital sodium (2.5%) was injected intraperitoneally for anaesthesia. In the sham group, only an open operation was performed to separate the mesentery of the distal caecum and large intestine, and the abdomen was closed. In the CLP group, the abdomen was disinfected routinely. The skin was cut approximately 1 cm apart, and the abdominal cavity was opened to search for the caecum. Then, the 1/2 distal of the caecum was tightly ligated with sterile No. 4 thread and punctured through the centre of the end of the ligated caecum with sterile No. 7 thread. The caecum was pushed back into the abdominal cavity, the abdominal cavity was closed, and the incision was sutured. The mice were sacrificed 12 h, 24 h or 48 h after the CLP operation, and the cerebral cortex tissues were collected.

### Immunofluorescence

Paraffin sections of cerebral cortex tissues were prepared, and the expression levels of NLRP3 and TLR4 were detected by an immunofluorescence analysis. After dewaxing and rehydration, the sections were placed in citrate buffer, and the antigens were recovered by a thermally mediated method. The sections were then incubated with 3% BSA for 30 min (Sigma–Aldrich, St. Louis, USA). Then, the sections were incubated overnight at 4 °C with diluted primary antibodies against NLRP3 (MA5–23919, Thermo Fisher Scientific) and TLR4 (ab22048, Abcam, Cambridge, UK). On the second day, the sections were incubated with a FITC-labelled secondary antibody at room temperature for 60 min. DAPI was used to stain the nuclei. Finally, the sections were dehydrated and examined under an inverted fluorescence microscope.

## Cell culture

The human microglial cell line CHME5 and embryonic kidney cell line 293 T were obtained from Fenghuibio (Changsha, Hunan, China). CHME5 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Wilmington, DE, USA) containing 10% foetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin–streptomycin. 293 T cells were cultured in DMEM (Thermo Fisher Scientific, Wilmington, DE, USA) supplemented with 10% FBS (Gibco), 2 mM L-glutamine, and 1% penicillin–streptomycin. All cells were cultured in an incubator with 5% CO<sub>2</sub> at 37 °C. Cell subculture was performed when the density reached 80–90%. To construct an in vitro inflammatory model, 100 ng/mL lipopolysaccharide (LPS) were applied to stimulate the CHME5 cells for 24 h as previously reported (Zhuang et al. 2020).

## Plasmid construction and transfection

MiR-25-3p mimics, TLR4 overexpression (OE-TLR4) vectors and respective negative controls (mimics NC and OE-NC) were all obtained from GenePharma (Shanghai, China). For the in vitro transfection, 100 nM indicated vectors were transfected into CHME5 cells using Lipofectamine™ 3000 transfection reagent in accordance with the manufacturer's instructions (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA). Subsequently, 48 h after the transfection, the efficiency was validated by qRT–PCR.

## Quantitative real-time polymerase chain reaction (qRT–PCR)

TRIzol reagent (Thermo Fisher Scientific) was employed to extract the total RNA. The RNA concentration and quality of each sample were determined with a Nanodrop Spectrophotometer (Thermo Fisher Scientific) using the 260/280 nm ratio. One microgram of RNA was synthesized into cDNA using a cDNA synthesis kit purchased from Yeason Biotech (Shanghai, China). The expression level of miR-25-3p was detected by a TaqMan miRNA Reverse Transcription Kit (Applied Biosystem, USA) as described in the manual. Following the cDNA synthesis, the cDNA was diluted to 5 ng/μL with pure water, and 5 μL diluted cDNA was subsequently used for the qRT–PCR assays using SYBR (QIAGEN, Germany). The primers used are shown in Table 1. The relative copy number of miR-25-3p, TLR4 and NLRP3 was calculated by the 2<sup>-ΔCt</sup> method, and the relative expression of TNF-α, IL-1β, IL-6 and IL-18 was calculated by the 2<sup>-ΔΔCt</sup> method. The U6 gene was used as an internal control for miR-25-3p. GAPDH was used as an internal control for the other genes.

## Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β, IL-6 and IL-18 in the tissue homogenate or cell culture supernatant were detected by ELISA. Tissue homogenates were collected and centrifuged at 3000 r/min for 15 min at 4 °C. Cells from different groups were collected and centrifuged at 1500 rpm for 5 min. Then, the supernatant was collected. ELISA kits were used to measure the levels of cytokines according to the manufacturer's instructions.

**Table 1** The primers sequences for qRT–PCR

	Forward (5'-3')	Reverse(5'-3')
M-miR-25-3p	CCGCATTGCACTTGTCTCG	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGAC TCAGAC
H-miR-25-3p	GCCCATTGCACTTGTCTCG	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGAC TCAGAC
M-Tlr4	GCCGGAAGGTTATTGTGGTA	AGGCGATACAATCCACCTG
H-TLR4	AGAAACTGCTCGGTCAGACG	AATGGAATCGGGGTGAAGGG
M-Nlrp3	CCATCAATGCTGCTTCGACA	GAGCTCAGAACCAATGCGAG
H-NLRP3	AAGGCCGACACCTTGATATG	CCGAATGTTACAGCCAGGAT
M-TNF-α	CCGATGGGTTGTACCTTGTC	TGGAAGACTCCTCCAGGTA
H-TNF-α	CCCCAGGGACCTCTCTCTAA	TGAGGTACAGGCCCTCTGAT
M-IL-1β	GCCACCTTTTGACAGTGATGAG	AAGGTCCACGGGAAAGACAC
H-IL-1β	CGATGCACCTGTACGATCAC	TCTTTCAACACGCAGGACAG
M-IL-6	TGCAAGAGACTTCCATCCAG	TCCACGATTTCCAGAGAAC
H-IL-6	ACAGGGAGAGGGAGCGATAA	GAGAAGGCAACTGGACCGAA
M-IL-18	GGCTGCCATGTCAGAAGACT	GGGTTCACTGGCACTTTGAT
H-IL-18	TGGCTGCTGAACCAGTAGAA	ATAGAGGCCGATTTCCTTGG

H represent human, M represent mouse

## Western blot analysis

The proteins were isolated with RIPA buffer, and the protein concentrations were determined by a BCA kit (Thermo Fisher Scientific). Proteins (20~30 µg) were separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Thermo Fisher Scientific). Then, the membranes were blocked with 5% nonfat milk. The membranes were incubated with primary antibodies at 4 °C overnight. On the next day, the membranes were incubated with a secondary antibody for 2 h at room temperature. After adding ECL, an imaging system (Chemiscope 6000, Clinx, Shanghai, China) was used to take photos of the bands. The following primary antibodies were used: IBA-1 (ab178846, 1:1000, Abcam), NLRP3 (ab263899, 1:1000, Abcam), TLR4 (ab13556, 1:1000, Abcam), caspase-1 (ab179515, 1:1000, Abcam), human ASC (ab151700, 1:1000, Abcam), mouse ASC (#67824, 1:1000, Cell Signaling Technology), AP-1 (#9165, 1:1000, Cell Signaling Technology), and GAPDH (#5174, 1:1000, Cell Signaling Technology). GAPDH was used as an internal reference gene.

## Dual luciferase assay

StarBase was used to predict the binding sites between miR-25-3p and TLR4. Mutated binding sites were generated by a QuikChange mutagenesis Kit (Stratagene). The fragments containing the predicted sequence of wild-type (WT) TLR4 or mutant (MUT) TLR4 were subcloned into the pGL3 vector (Promega, Madison, WI, USA). All constructed plasmids were sequenced for verification. The constructed pGL3-TLR4-WT or pGL3-TLR4-MUT vector was transfected into 293 T cells with mimics NC or miR-25-3p mimics. Then, the relative luciferase activity was analysed.

## Statistical analysis

The mean ± standard deviation (SD) is used to present the data. The differences between two groups were analysed by Student's *t* test. A one-way analysis of variance (ANOVA) with Tukey's post-hoc test was employed to evaluate the differences among multiple groups.  $P < 0.05$  was defined as statistically significant.

## Results

### MiR-25-3p was decreased while TLR4 and NLRP3 were upregulated in the SAE mouse model

We first established an SAE mouse model to evaluate the expression of miR-25-3p, TLR4 and NLRP3. At 12 h, 24 h or 48 h after the CLP operation, the mice were sacrificed, and the cerebral cortex tissues were collected. As expected,

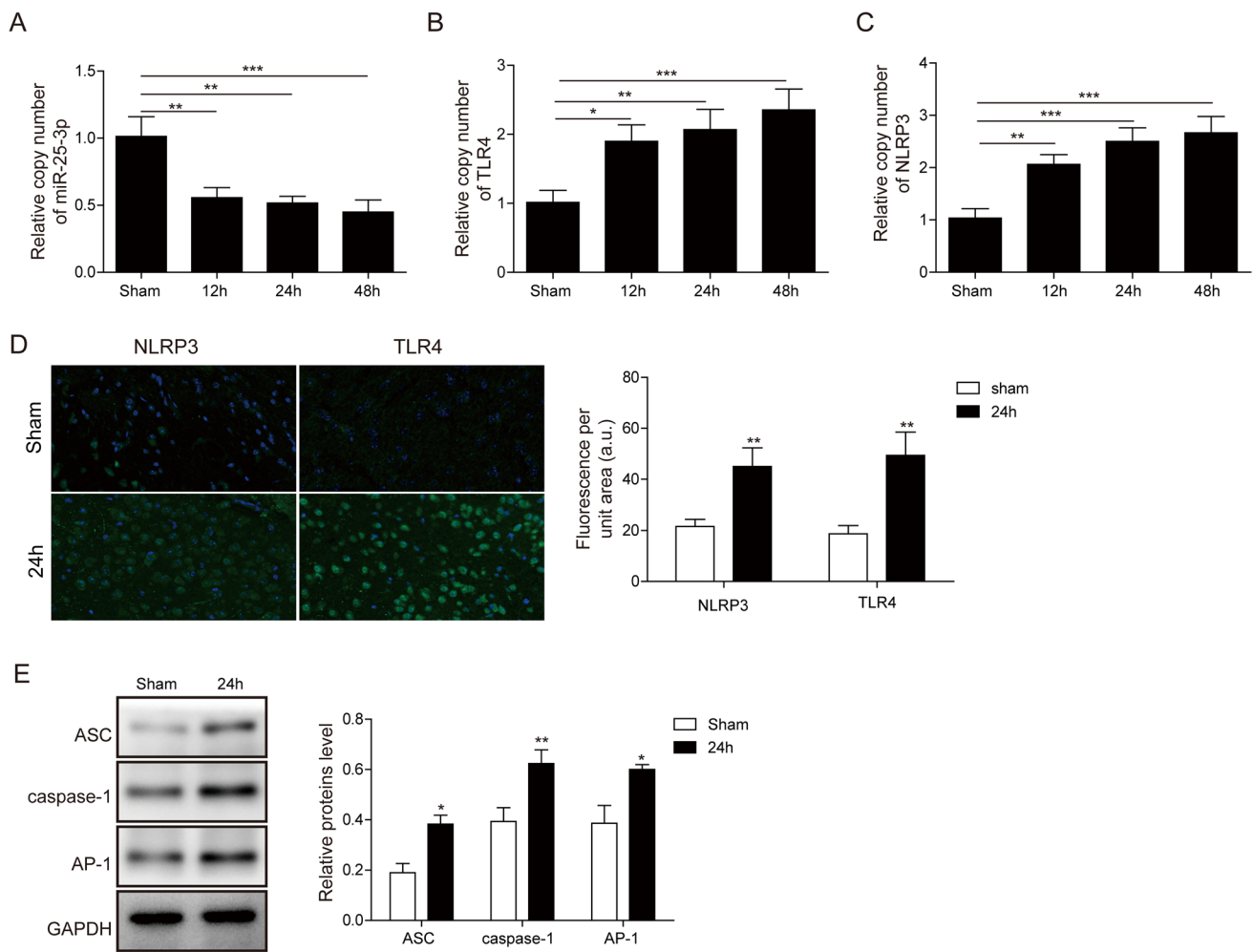
the relative copy number of miR-25-3p in the mouse cerebral cortex tissues was reduced by the CLP operation in a time-dependent manner (Fig. 1A), and the TLR4 and NLRP3 relative copy numbers were notably increased (Fig. 1B and C). Subsequently, we detected the levels of TLR4 and NLRP3 in the cortical region of SAE by immunofluorescence, and the results were consistent with the qRT-PCR results (Fig. 1D). Next, we investigated the levels of inflammasome-relevant proteins, including ASC, caspase-1 and AP-1. The results showed that the ASC, caspase-1 and AP-1 levels were significantly increased by the CLP operation in a time-dependent manner (Fig. 1E). Collectively, these results confirmed that CLP induced the activation of the NLRP3 inflammasome and the level of TLR4 while downregulating miR-25-3p expression in the SAE mouse model.

### Relevant cytokines were upregulated in the SAE mouse model

Next, we examined the cytokine levels in mouse cerebral cortex tissues 12 h, 24 h or 48 h after the CLP operation. As shown in Fig. 2A-D, the mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 in the mouse cerebral cortex tissues were increased by the CLP operation in a time-dependent manner. Additionally, similar trends were observed in the serum levels of cytokines (Fig. 2E-H).

### Overexpression of miR-25-3p suppressed LPS-induced activation of microglia

Subsequently, we applied an in vitro inflammatory model by stimulating CHME5 cells with LPS to confirm the expression pattern of the indicated genes in SAE. As shown in Fig. 3A, the relative copy number of miR-25-3p was reduced in the LPS-treated CHME5 cells, whereas the TLR4 and NLRP3 relative copy numbers were increased. To unveil the functional significance of miR-25-3p in LPS-induced microglial activation, miR-25-3p mimics were transfected into CHME5 cells. The transfection efficiency was validated by qRT-PCR, and the results showed that the miR-25-3p relative copy number was significantly increased by miR-25-3p mimics in CHME5 cells. The western blot analysis results demonstrated that the LPS stimulation resulted in a significant increase in IBA-1, TLR4, NLRP3, ASC, caspase-1 and AP-1, while miR-25-3p overexpression inhibited the expression of these proteins in the LPS-treated CHME5 cells (Fig. 3C). Collectively, these results indicated that the overexpression of miR-25-3p resulted in the downregulation of inflammatory cytokine expression and protein production in LPS-treated CHME5 cells.



**Fig. 1** miR-25-3p was downregulated, while TLR4 and NLRP3 were upregulated in the SAE mouse model. CLP operation was employed to induce SAE in mice, the mice were sacrificed, and the cerebral cortex tissues were collected 12 h, 24 h or 48 h after the CLP operation. (A–C) The relative copy numbers of miR-25-3p (A), TLR4 (B) and

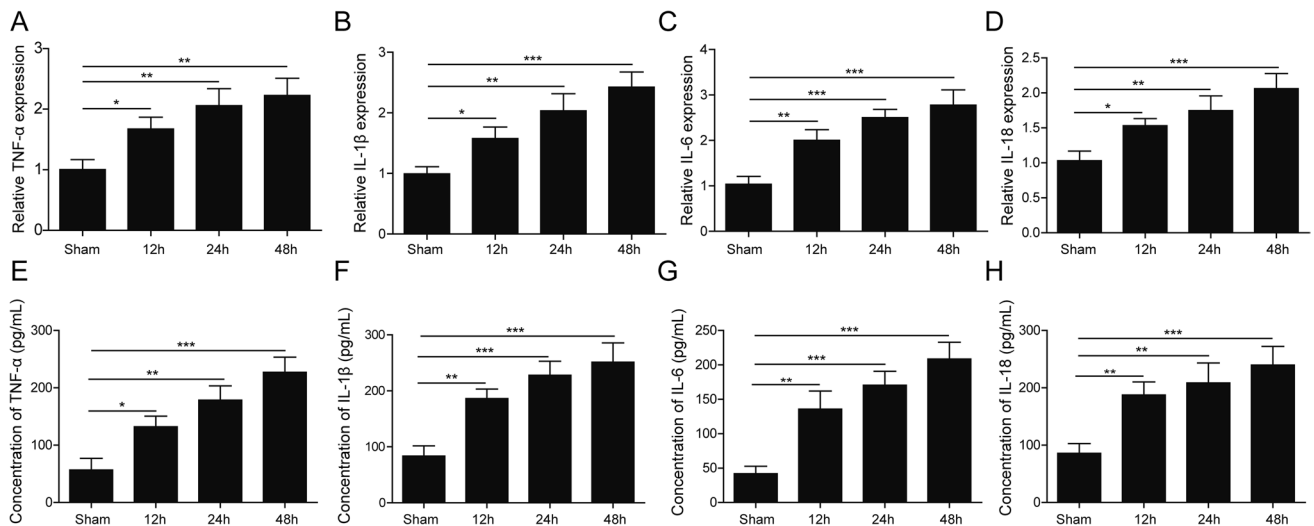
NLRP3 (C) were determined by qRT-PCR. (D) The expression of TLR4 and NLRP3 was examined by immunofluorescence in cerebral cortex tissues from SAE mice. (E) The protein levels of ASC, caspase-1, and AP-1 were determined by a western blot analysis. N=3. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

### miR-25-3p overexpression suppressed LPS-induced inflammatory cytokine expression

Additionally, we found that the LPS stimulation induced the mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 in CHME5 cells. However, the levels of these cytokines were suppressed by miR-25-3p overexpression (Fig. 4A–D). Similarly, we performed ELISA to detect the levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 in the supernatant, and the results indicated that the expression of these cytokines was elevated after the LPS stimulation; however, the overexpression of miR-25-3p reversed the increase in cytokine expression induced by LPS (Fig. 4E–H). Taken together, we found that miR-25-3p overexpression suppressed LPS-induced cytokine expression.

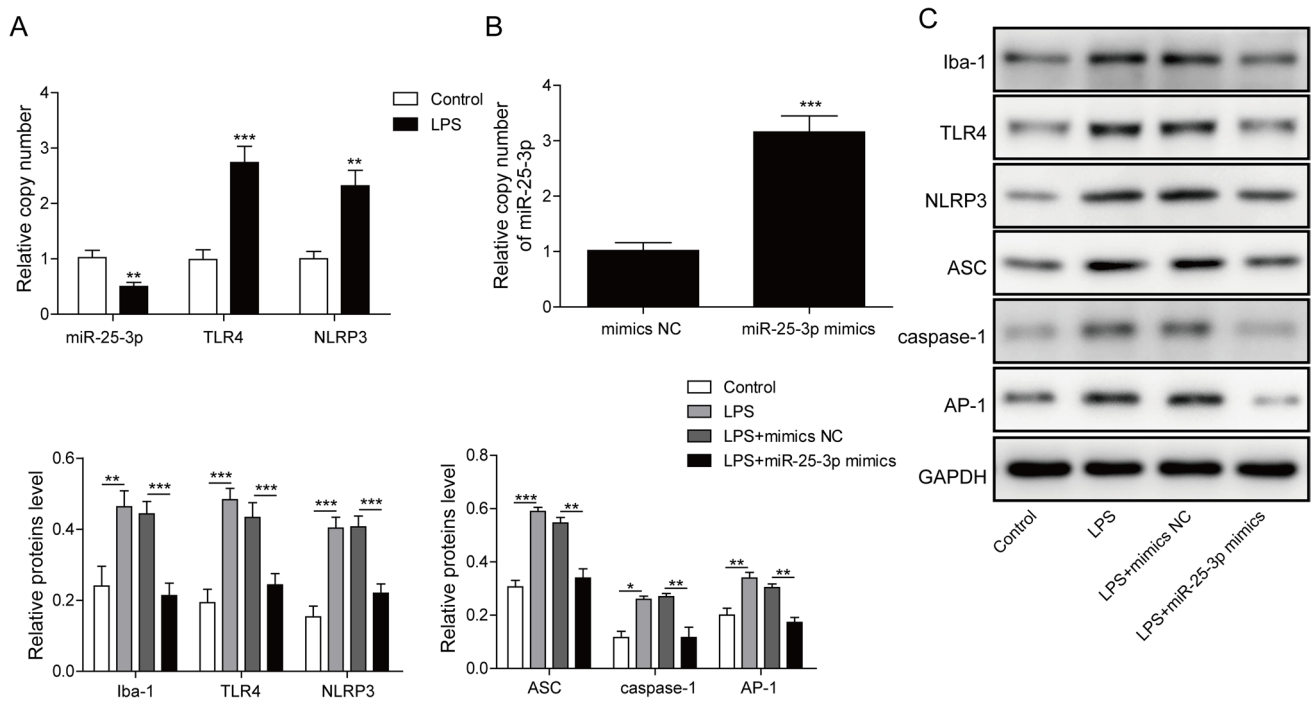
### Overexpression of TLR4 eliminated the effect of miR-25-3p overexpression in LPS-stimulated CHME5 cells

Next, we investigated the downstream target of miR-25-3p. As shown in Fig. 5A, there was a potential binding site between miR-25-3p and TLR4. The relative luciferase activity was significantly reduced after the cotransfection of miR-25-3p mimics with a fragment containing TLR4 wild-type binding sites; however, the TLR4-mut vector and miR-25-3p mimics cotransfection had no significant effect on the relative luciferase activity (Fig. 5B). Moreover, as shown in Fig. 5C and D, the miR-25-3p overexpression substantially repressed the expression of TLR4 and NLRP3. To investigate whether miR-25-3p plays a key role by regulating TLR4, we



**Fig. 2** Relevant cytokines were upregulated in the SAE mouse model. CLP operation was employed to induce SAE in mice, the mice were sacrificed, and the cerebral cortex tissues were collected 12 h, 24 h or 48 h after the CLP operation. (A–D) The mRNA levels of TNF- $\alpha$ ,

IL-1 $\beta$ , IL-6 and IL-18 were evaluated by qRT-PCR. (E–H) The levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 were evaluated by ELISA. N=3. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

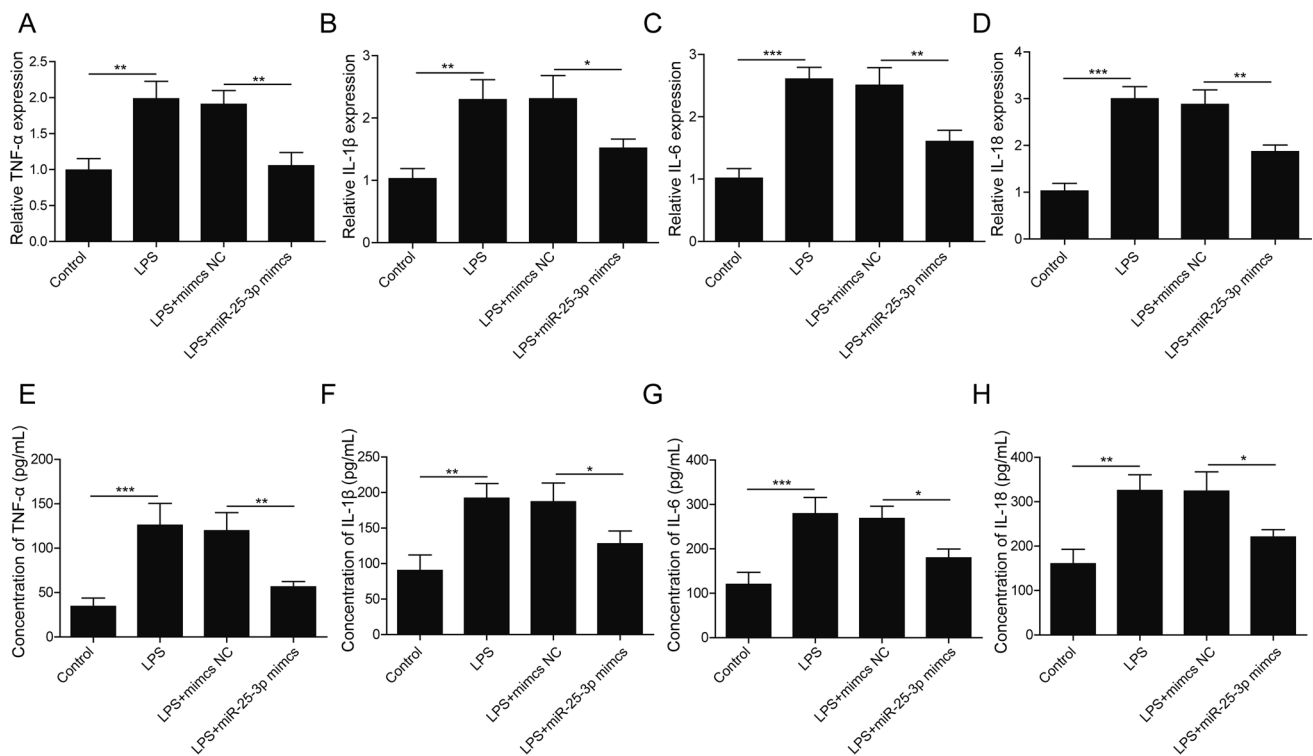


**Fig. 3** Overexpression of miR-25-3p suppressed LPS-induced activation of microglia. (A) CHME5 cells were stimulated with 100 ng/mL LPS for 24 h, and the relative copy numbers of miR-25-3p, TLR4, and NLRP3 were determined by qRT-PCR. (B) MiR-25-3p overexpression vector was transfected into CHME5 cells. The relative copy

number of miR-25-3p was determined by qRT-PCR. (C) CHME5 cells were transfected with miR-25-3p mimics and then stimulated with 100 ng/mL LPS for 24 h. The protein levels of IBA-1, TLR4, NLRP3, ASC, caspase-1 and AP-1 were determined by a western blot analysis. N=3. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

overexpressed TLR4 in CHME5 cells. The qRT-PCR and western blot analyses demonstrated that TLR4 overexpression promoted the mRNA and protein levels of TLR4

and NLRP3 (Fig. 5E and F). The miR-25-3p overexpression inhibited the expression of IBA-1, TLR4, NLRP3, ASC, caspase-1 and AP-1 under LPS stimulation.



**Fig. 4** Overexpression of miR-25-3p suppressed LPS-induced inflammatory cytokine expression. CHME5 cells were transfected with miR-25-3p mimics and then stimulated with 100 ng/mL LPS for 24 h. (A–D) The mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 were eval-

uated by qRT-PCR. (E–H) The concentrations of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 were evaluated by ELISA. N=3. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

However, the TLR4 overexpression reversed the inhibitory effects of miR-25-3p on the expression of these proteins (Fig. 5G). Taken together, the above results confirmed that miR-25-3p overexpression inhibited the inflammatory response in LPS-stimulated CHME5 cells by directly targeting TLR4.

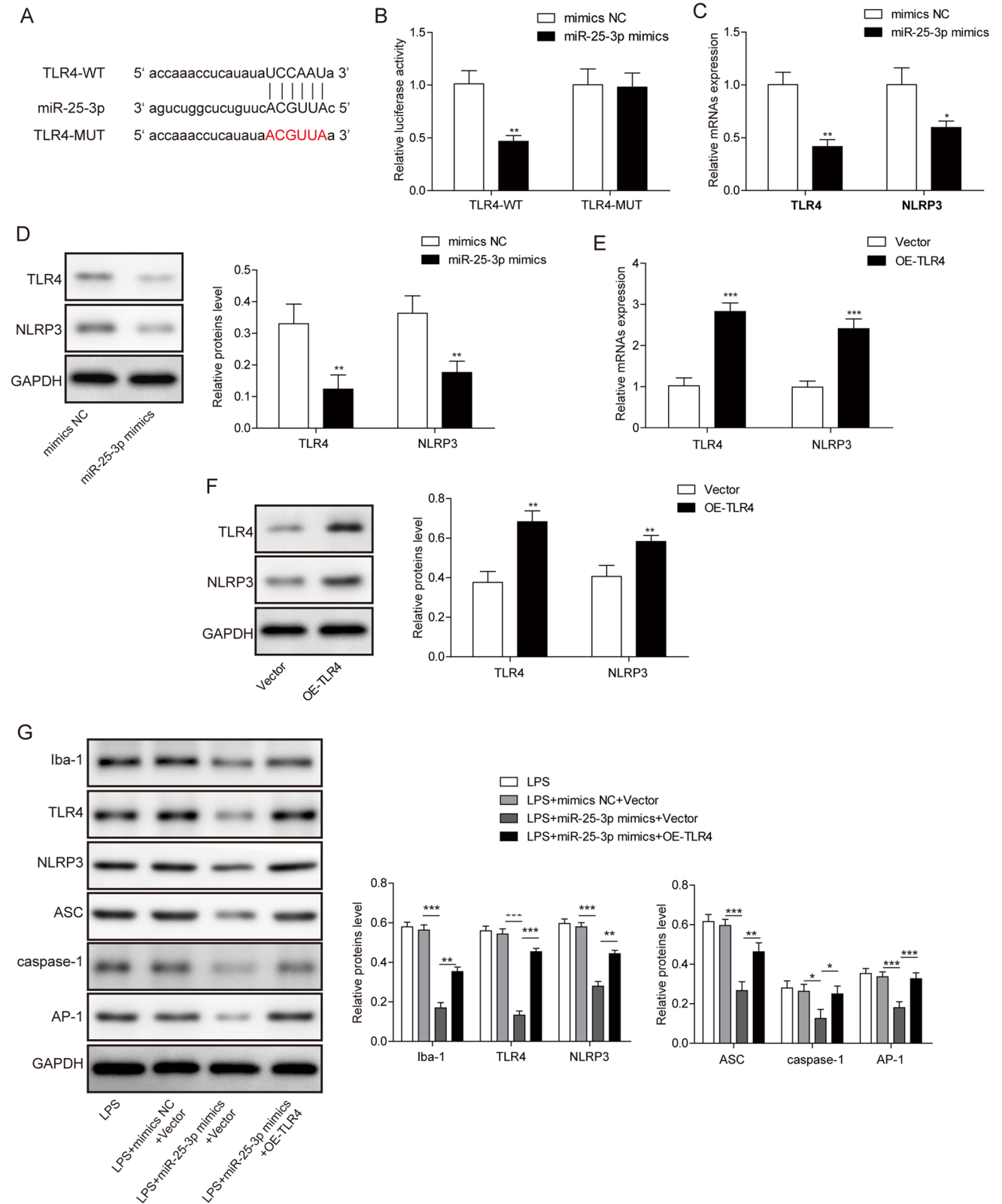
### Overexpression of TLR4 reversed the effect of miR-25-3p overexpression on inflammatory cytokine secretion

The qRT-PCR results showed that the miR-25-3p mimics reduced the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 in LPS-treated CHME5 cells. However, the TLR4 overexpression also reversed the inhibitory effect of miR-25-3p on the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 (Fig. 6A–D). Consistent with the above results, the ELISAs showed that the miR-25-3p overexpression inhibited the levels of cytokines, while the cotransfection of OE-TLR4 and miR-25-3p mimics promoted the release of these cytokines in the supernatant (Fig. 6E–H). Taken together, these results indicated that the overexpression of TLR4 alleviated the effect of miR-25-3p overexpression on cytokine release.

## Discussion

SAE is a common complication of sepsis that may seriously affect the prognosis and quality of life of patients with sepsis (Yin et al. 2020). Microglial activation is vital for neuroinflammation and the pathology of SAE (Wen et al. 2021). In the present study, we found that the expression of miR-25-3p was decreased and that microglia were excessively activated in SAE mice. Furthermore, the overexpression of miR-25-3p attenuated LPS-induced microglial activation by targeting the TLR4/NLRP3 axis.

Accumulating studies show that miRNAs are involved in regulating the occurrence and development of SAE. For example, Visitchanakun et al. illustrated that miR-370-3p in plasma served as a marker of SAE and that miR-370-3p overexpression remarkably promoted TNF- $\alpha$ -induced neuronal apoptosis (Visitchanakun et al. 2020). In addition, miR-181b aggravated blood–brain barrier impairment by targeting SP1 (Chen et al. 2020). In the present study, our results revealed the functional significance of miR-25-3p in SAE. It was found that the expression level of miR-25-3p was significantly reduced in the cerebral cortex of SAE mice. Moreover, the miR-25-3p overexpression suppressed LPS-induced microglial activation and the inflammatory



response. These results are consistent with the protective role of miR-25-3p revealed in previous studies (Yao et al. 2018; Zhu et al. 2018). Collectively, platelet-derived exosomal

miR-25-3p could alleviate oxidized low-density lipoprotein-induced coronary vascular endothelial cell inflammation by inhibiting the expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$



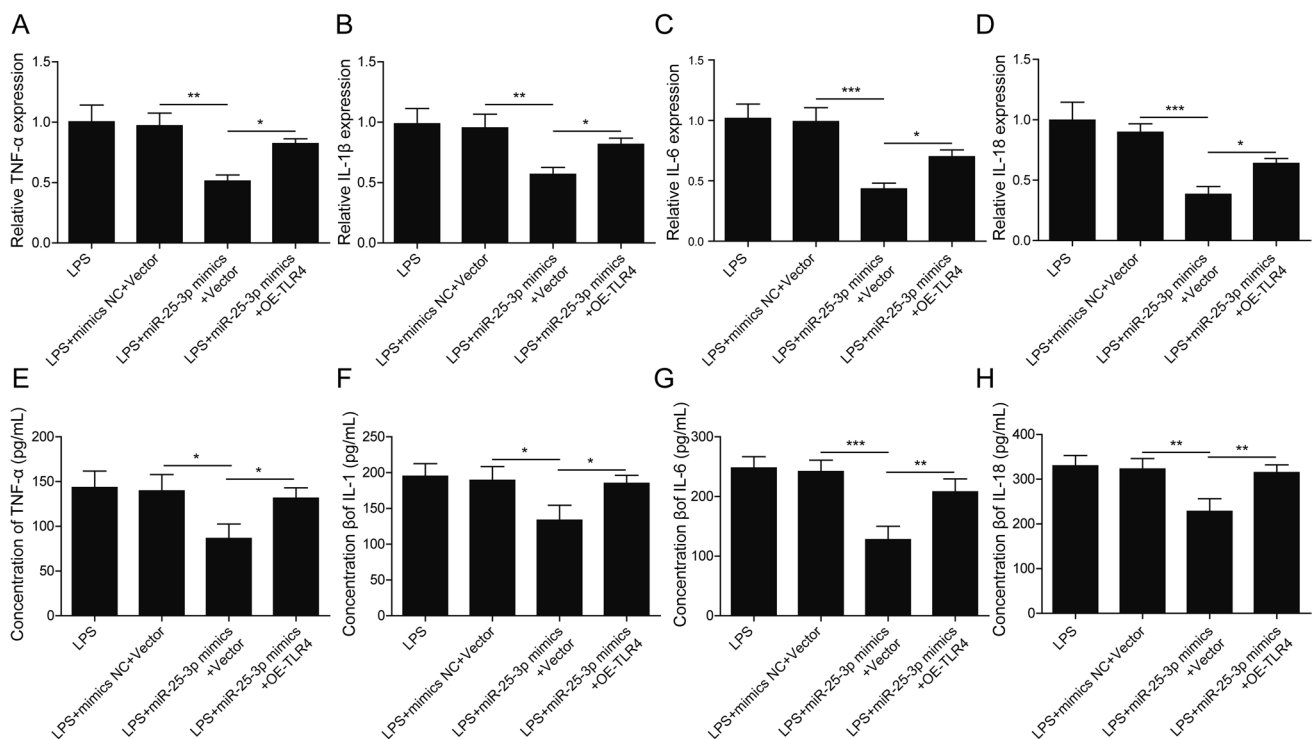
**Fig. 5** Overexpression of TLR4 eliminated the effect of miR-25-3p overexpression in LPS-stimulated CHME5 cells. **(A)** Illustration of the binding site between miR-25-3p and TLR4. **(B)** The relative luciferase activity was validated by a dual luciferase reporter assay. **(C)** The mRNA levels of TLR4 and NLRP3 were examined by qRT-PCR. **(D)** The protein levels of TLR4 and NLRP3 were examined by a western blot analysis. **(E)** The mRNA levels of TLR4 and NLRP3 were examined in CHME5 cells after transfection with TLR4 overexpression vectors by qRT-PCR. **(F)** The protein levels of TLR4 and NLRP3 after TLR4 overexpression were examined by a western blot analysis. **(G)** CHME5 cells were cotransfected with TLR4-overexpressing and miR-25-3p mimics, followed by LPS stimulation. The protein levels of IBA-1, TLR4, NLRP3, ASC, caspase-1 and AP-1 were determined by a western blot analysis. N=3. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

in ApoE<sup>-/-</sup> mouse models of atherosclerosis (Yao et al. 2019). In our study, we found that miR-25-3p overexpression reduced the release of inflammatory cytokines, suggesting that miR-25-3p had a protective effect in SAE.

TLR4 is a key receptor responsible for LPS-induced inflammatory disorders (Kuzmich et al. 2017). TLR4 plays vital roles in the pathogenesis of sepsis; thus, TLR4 may be a potential target for the amelioration of inflammation in sepsis (Venancio et al. 2016). TLR4 knockdown remarkably repressed the inflammatory response in sepsis (Zhang and Niu 2019). MiRNAs regulate the pathological process

of many biological cells through the degradation of target genes or posttranscriptional inhibition of target gene translation (Afonso-Grunz and Muller 2015). It was also reported that miR-140-5p alleviated sepsis-induced lung injury by targeting TLR4 (Lin et al. 2020). In the present study, we found that miR-25-3p targeted TLR4 to inhibit TLR4 expression and that TLR4 overexpression eliminated the inhibitory effect of miR-25-3p on the inflammatory response in LPS-treated CHME5 cells. Previous studies revealed that TLR4 signalling resulted in NLRP3 inflammasome activation in several diseases, including coronary microembolization-induced myocardial injury (Su et al. 2018) and contrast-induced acute kidney injury (Tan et al. 2017). The results of the present study also suggested that TLR4 induced the NLRP3 inflammasome, which was consistent with previous studies. In summary, miR-25-3p played a protective role in sepsis and sepsis complications by targeting TLR4/NLRP3.

In conclusion, miR-25-3p expression was reduced in LPS-induced CHME5 and SAE mice, and the overexpression of miR-25-3p suppressed the expression of inflammatory cytokines, suggesting that miR-25-3p played a protective role in SAE. Mechanistically, miR-25-3p attenuated the activation of microglia by inhibiting the NLRP3/IL-1 $\beta$ /IL-18 axis by directly targeting TLR4. Our study might provide a novel molecular target for the treatment of SAE.



**Fig. 6** Overexpression of TLR4 reversed the effect of miR-25-3p overexpression on inflammatory cytokine secretion. CHME5 cells were cotransfected with a TLR4-overexpressing plasmid and miR-25-3p mimics and then stimulated with LPS. (A-D) The mRNA lev-

els of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 were evaluated by qRT-PCR. (E-H) The concentration levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18 were evaluated by ELISA. N=3. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

**Abbreviations** SAE: Sepsis-associated encephalopathy; TLR4: Toll like receptor 4; NLRP3: NOD-like receptor family members 3; microRNA: miRNA; 3'UTR: 3' translational region; TNF- $\alpha$ : Tumor necrosis factor  $\alpha$ ; IL-1 $\beta$ : Interleukin 1 $\beta$ ; IL-6: Interleukin 6; IL-18: Interleukin 18; qRT-PCR: Reverse transcription followed by quantitative real-time PCR; WB: Western blots

**Authors' contributions** Xiao-Yan Luo: Conceptualization, Methodology, Data curation, Visualization, Investigation, Validation, Software, Writing- Reviewing and Editing; Jian-Hua Ying: Visualization, Investigation, Validation, Software; Qiao-Sheng Wang: Data curation, Writing- Original draft preparation, Writing- Reviewing and Editing. All authors read and approved the final manuscript.

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**Data availability** The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Ethics approval and consent to participate** All in vivo experiments were performed in accordance with National Institutes of Health guide for the care and use of laboratory animals, following a protocol approved by the Ethics Committees of the First Affiliated Hospital of University of South China.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare that they have no conflict of interest.

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