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RESEARCH

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Long non-coding RNA Tug1 regulates inflammation in microglia and in status epilepticus rats through the NF-κB signaling pathway

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Abstract

Background: Inflammation plays an important role in the pathogenesis of status epilepticus (SE). The long non-coding RNA (lncRNA) taurine up-regulated gene1 (Tug1) plays a well-defined role in inflammatory diseases. However, the molecular mechanism of Tug1 in SE progression remains unknown. In present study, we investigated whether Tug1 is involved in microglial inflammation in SE rats.

Methods: The SE rat model was established via intraperitoneal injection of lithium chloride-pilocarpine. RNA-binding protein immunoprecipitation (RIP) and RIP sequencing were carried out in rat microglia (RM). Tug1 cloned into the adenovirus was overexpressed in the microglia. Knockdown of Tug1 was performed via siRNA transfection. The level of Tug1 and inflammatory factors IL-1β and TNF-α was examined by real-time polymerase chain reaction (RT-PCR) and western blotting. Protein levels of p65, p-p65, p-IκBα and IκBα were assessed by western blotting.

Results: The RIP-seq result showed 14 lncRNAs that bound to the NF-κB p65 protein in RM. The lncRNA Tug1 directly interacted with p65. The level of declined Tug1 was decreased in the hippocampus of SE rats. Overexpression of Tug1 reduced the LPS-induced inflammation and M1/M2 polarization of microglia, while knockdown of Tug1 aggravated the inflammatory response in microglia. Accordingly, the protein levels of p-p65/p65 and p-IκBα/IκBα were reduced in the Tug1-overexpression microglia and elevated in the Tug1-knockdown microglia.

Conclusions: These findings indicate that Tug1 modulates the inflammation in microglia through the NF-κB signal pathway, and the Tug1/P65 axis are like to play a significant role in the inflammatory processes, providing a valid target for the therapy of SE.

Keywords: Inflammation, Microglia, Status epilepticus, lncRNA, NF-κB signaling pathway, Tug1

Background

Status epilepticus (SE) is a common neurological emergency characterized by high mortality[1]. The progression of SE is considered to be closely related to inflammation[2]. Microglia are one type of glial cells with inflammatory functions and contribute to various brain pathologies[3]. Numbers studies have confirmed that microglia are activated in brains of SE, causing a rapid and intense inflammatory cascade[4]. The microglia-mediated inflammation has a major role in status epilepticus[5]. The NF-κB signaling pathway is an important inflammatory pathway[6]. Inhibition of the NF-κB pathway has been demonstrated to attenuate the

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neuroinflammation in the brains of SE rats and attenuate corresponding damage[7, 8]. However, exogenous inhibitors of NF-κB signaling pathway have limited clinical application due to their toxicity and non-specificity[9]. Therefore, seeking molecules that can endogenously regulate the NF-κB signaling pathway may help solve this problem. Long non-coding RNAs (lncRNAs) are characterized as untranslated RNAs greater than 200 nt in length[10]. As an endogenous small molecule, lncRNAs have a strong gene-regulation ability. Recently studies have been reported that the lncRNAs abnormally expressed in neurological disorders and participate in variety of inflammatory responses[11, 12]. Increasing evidence has suggested that lncRNAs could inhibit inflammation through the NF-κB signaling pathway[13, 14]. Therefore, determining the specific lncRNA(s) that regulate the NF-κB signaling pathway in microglia will help effectively intervene inflammation in the brain.

In this study, we screened for lncRNAs directly binding to NF-κB p65 in microglia, and selected one lncRNA (Tug1) for in vitro validation. The expression of the lncRNA Tug1 in rat hippocampus and cultured microglia was confirmed by RT-PCR. Overexpression or knockdown of Tug1 was performed in cultured microglia to verify the mechanism of lncRNA in the NF-κB signaling pathway and inflammation.

Materials and methods

Cell culture

Rat microglia (RM) were purchased from ScienCell (R1900, USA). Microglia were grown in high glucose medium with 10% fetal bovine serum and cultured in a 37℃ cell incubator with 5% CO_2. When microglia were 65%-70% confluent, the microglia were stimulated with LPS (10 μg/ml). Total RNA and protein were extracted 48 h post-treatment.

RNA immunoprecipitation (RIP) and RNA sequencing

RIP was carried out using an Immunoprecipitation kit (Millipore, USA) as described in the product instructions. The cell lysate was initiated with anti-P65 antibody (Abcam, USA) or isotype control IgG (Abcam,USA). The RNA-protein complexes were precipitated with protein A magnetic beads. The U1 small nuclear RNA (snRNA) served as the negative control snRNA of Tug1. The immunoprecipitated RNA was isolated using Trizol (Takara, Japan) and further RT-PCR analysis was performed. The purified RNA was sequenced.

Rat model of SE

Male SD rats aged 14 days were raised in the Experimental Animal Center of Shanghai Public Health Clinical Center. 24 h post-injection of pilocarpine (20 mg/kg, i.p.), the rats were given 127.3 mg/kg lithium chloride (i.p.). And 1 mg/kg scopolamine was injected intraperitoneally 30 min before pilocarpine. Status epilepticus (SE) was scored on the Racine scale. After 60 min of SE, the convulsion was terminated with 10 mg/kg diazepam.

Cell transfection

To overexpress Tug1, full-length Tug1 was cloned into the adenovirus vector (HBAD-r-Tug1-Null-EGFP). Then the RM were transfected with HBAD-r-Tug1-Null-EGFP (OE-Tug1) adenovirus or negative control (OE-NC) empty HBAD-EGEP adenovirus using LipofectamineTM 2000 (ThermoFisher, USA). Small interfering RNA of lncRNA Tug1 (si-Tug1) was designed by GenePharma company (Shanghai, China) to knock down Tug1. The negative control siRNA (si-NC) was used as negative control. The above primer sequences are given below: si-Tug1: sense 5'-GGUCUUCUACCCCU-3'; antisense 5' UACCGAUGCAGAAUAGAAGC; si-NC: sense 5'-GGU CCCGAACGUGUCACG-3'; antisense 5'-AUGCGC CAUGCU-3′. The siRNAs were transfected using LipofectamineTM 2000 (ThermoFisher, USA) according to the instruction manual.

Western blotting

Proteins of rat hippocampal tissues and microglia were extracted by using 1% PMSF. The SDS-PAGE proteins (15-20 μg) were separated and transferred to membrane. The PVDF membrane were blocked with blocking solution for 30 min. And incubated with the primary antibody for p-p65, p65, p-IκBα, IκBα, IL-1β or TNF-α overnight rotation at 4 degrees. HRP-conjugated secondary antibody were incubated at room temperature. Blot bands were developed with ECL kit and quantified using the ImageJ software (USA). GAPDH served as the normalization control for quantification of the proteins.

RT-PCR

Rat hippocampal and cells were treated with Trizol (Takara, Japan). Tissue was homogenized by a tissue homogenizer. Chloroform and isoamyl alcohol were used to extract mRNA. RNA concentration was quantified using spectrophotometer and the Prime Script reagent kit (Takara, Japan) was used for reverse transcription. PCR reactions was carried out using the PCR kits (Takara, Japan). The mRNA expression level was normalized to GAPDH. The primers sequences are given below: Tug1 (5′-CCGGATTAACACCAAGGAAG-3′) and (5′-TTAGCGGGCCATATA-3′), GAPDH (5′-TTCGCCATG GATATC-3′ and 5′-TAGGAGTCCTTCTATAC-3′), and IL-β (5′-CACACTAGCAGG TCATCC-3′ and 5′-ATC TCAC AGCAGCATCTCGACAAG-3′).

Immunofluorescence staining

Paraformaldehyde (4%) was used to fix the microglia. Cells were permeabilization with 0.1% Triton (ThermoFisher, USA) for 15 min and followed by blocking with 1% BSA at room temperature for at least 1 h. Primary antibody incubations were performed in microglia overnight at 4-degrees. Secondary antibodies and DAPI were incubated after TBST wash. Stained cells were observed using fluorescence microscopy.

Statistical analysis

All data analyses were analyzed using GraphPad Prism 7.0 statistical software. Comparisons between groups were carried out using one-way ANOVA or *t*-test. Pearson's correlation analysis to determine p and r values. *P* value of less than 0.05 was considered statistically significant. Unless otherwise stated, all experiments were repeated three times with three replications each.

Results

LncRNA Tug1 interacted with NF-κB directly

To screen lncRNAs that bind to NF-κB p65, we first performed the RIP-seq in microglia. Results showed that there were 2557 mRNAs and 22 non-coding RNAs binding to NF-κB p65, including 8 miRNAs and 14 lncRNAs (Fig. 1a). The enrichment expression of p65-binding lncRNAs is presented in Fig. 1b, and lncRNA Tug1 was selected for further studies according to previous studies. The result of RIP showed enrichment of Tug1 in the P65 pull-down (Fig. 1c). Consistently, POSTAR2 analysis showed that Tug1 bound to P65 directly (Fig. 1d), predicting a sequence motif for NF-κB p65 binding.

Tug1 was downregulated in the hippocampus of SE rats

Tug1 expression showed a clear trend of decrease in SE rats (Fig. 2a). The expression of Tug1 increased slightly 6 h after SE compared to the normal group,

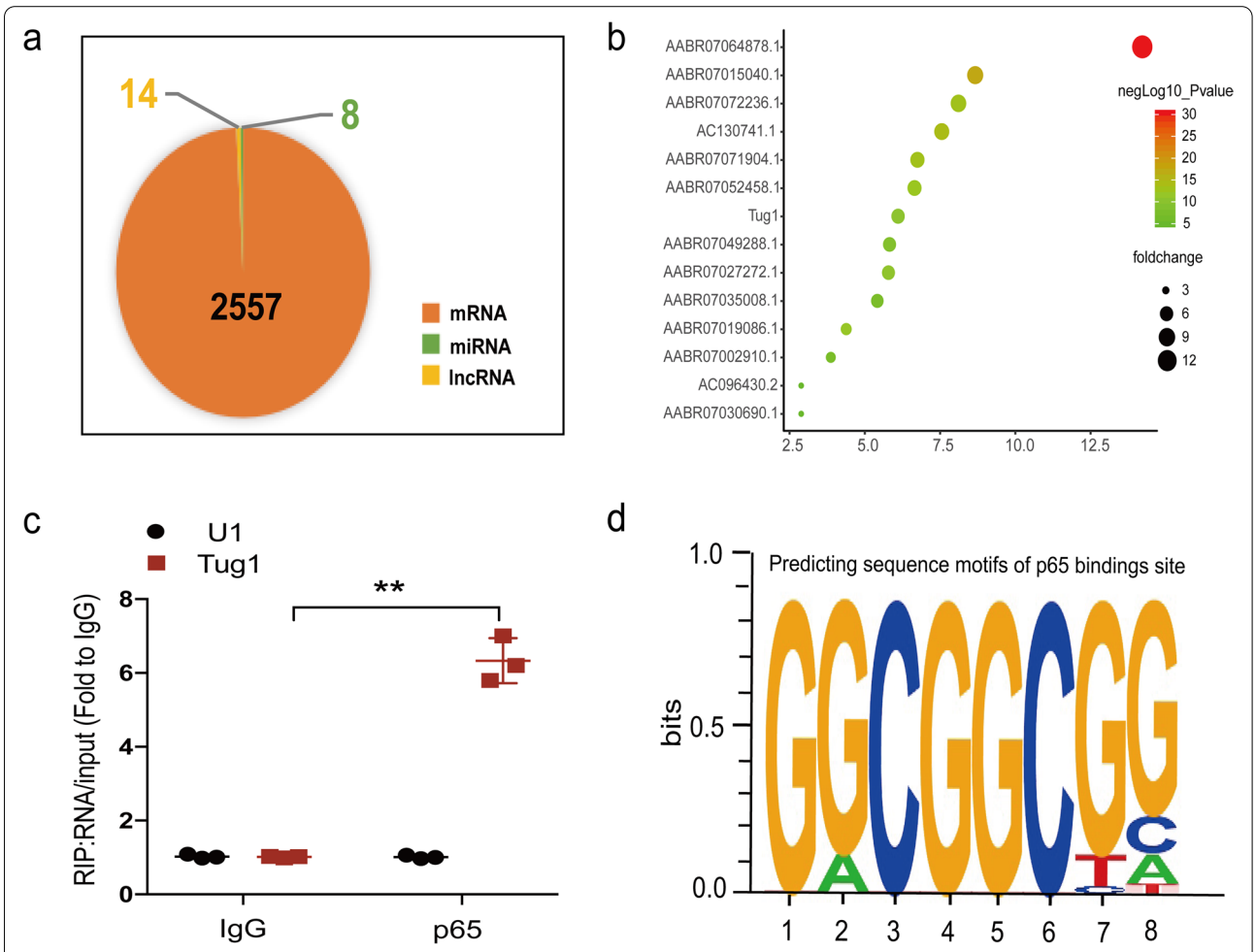

Fig. 1 The data of RIP sequencing in microglia. **a** The number of RNAs binding to the NF-κB p65 in microglia. **b** The bubble diagram of long non-coding RNAs binding to the NF-κB p65 protein. **c** RIP assays of Tug1 in microglia. **d** POSTAR2 was used to predict the sequence motifs of p65-binding sites. Student's *t* test was used (**$P < 0.01$ vs. IgG)

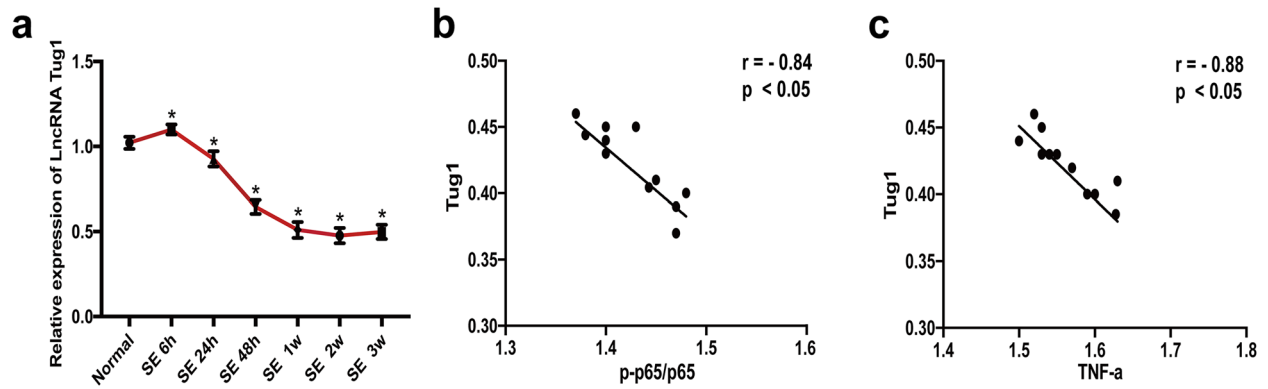

Fig. 2 Tug1 was downregulated in the hippocampus of SE rats. **a** The level of Tug1 in rat hippocampus after SE ($n = 3$-4). **b** Pearson correlation analysis of Tug1 and p-p65/p65 in hippocampus of 2-week group. **c** Pearson correlation analysis of Tug1 and TNF-α mRNA in rat of SE 2-week group

and decreased rapidly within 48 h after SE. There was a negative relationship with Tug1 level and p-p65/p65 ratio (Fig. 2b), indicating a relationship of Tug1 expression with the NF-κB signaling pathway. In addition, Tug1 expression was negatively correlated with inflammatory factor TNF-α (Fig. 2c). The findings suggest that the low expression of Tug1 in SE rats related to the NF-κB signaling pathway and inflammation.

Tug1 was downregulated in LPS-induced microglia.

Microglia culture are a powerful in vitro tool to study the specific molecular pathways involved in neuroinflammation. Thus, we used LPS-induced inflammation in microglia that partly mimics the SE-induced neuroinflammation to explore the role of Tug1 in inflammation. Fluorescence microscopy showed strong expression of microglial markers Iba1 and F4/80 (Fig. 3a). These data suggest that the RM cells formed a pure population. The level of TNF-α and IL-1β were increased by LPS treatment at 1-10 μg/ml (*$P < 0.05$ vs Control, Fig. 3b). LPS treatment at 10 μg/ml upregulated the level of microglial M1 markers (CD11b, CD86, iNOS, IL-1β and TNF-α) and down-regulated the level of microglial M2 markers (IL-4 and IL-10, CD32, CD163, Arginase-1) in comparison to control (*$P < 0.05$, Fig. 3c, d). The level of Tug1 was decreased in the 10 μg/ml LPS stimulated microglia (**$P < 0.01$ vs Control, Fig. 3e). Further Pearson's correlation analysis showed a negative correlation of Tug1 with TNF-α and IL-1β (Fig. 3f, g). These results provide important insights into the hypothesis that Tug1 regulates inflammation in LPS-treated microglia.

Up-regulated Tug1 reduced inflammation through the NF-κB signaling pathway in microglia

To demonstrate further the function for Tug1 in LPS-treated microglia, we used adenovirus as a vector to overexpress Tug1. As shown in Fig. 4a, at adenovirus multiplicity of infection (MOI) of 30, the microglia had good morphology, and few deaths were observed. The result of PCR showed highest transfection efficiency at MOI of 30 (*$P < 0.05$ versus control, Fig. 4b). Thus, we chose a virus concentration at MOI of 30 for microglial infection. In Fig. 4c, the expression of Tug1 decreased in LPS treated microglia (*$P < 0.05$ vs Control), while Tug1 was successfully overexpressed in the LPS-induced microglia (differential fold > 2). LPS stimulated elevated the levels of p-65 and p-IκBα (*$P < 0.05$ *vs* control, Fig. 4f-h) in microglia. Conversely, overexpression of Tug1 reduced the level of p-65 and p-IκBα in LPS stimulated microglia (#$P < 0.05$). Consistent with this, the expression of inflammatory factors (TNF-α and IL-1β) was decreased in the LPS + OE-Tug1 group c (#$P < 0.05$ versus NC). In addition, overexpression of Tug1 dampened M1 polarization and triggered M2 polarization (Fig. 4d, e). The expression of M1 surface marker CD86 and iNOS was down-regulated (#$P < 0.05$ *vs* control) and M2 surface marker CD163 and arginase-1 were up-regulated in the OE-Tug1 set (#$P < 0.05$). The above results indicated that Tug1 suppressed the inflammatory response via regulating the NF-κB signaling pathway in microglia.

Knockdown of Tug1 aggravated inflammation in microglia

To further verify that Tug1 has an inhibitory effect on LPS-induced inflammation, we performed a reverse verification. As Fig. 5a, the level of Tug1 in the si-Tug1 group was down-regulated in contrast to the NC set ($P < 0.01$). Down regulation of Tug1 favors the M1 polarization and suppressed the M2 polarization. The expression of M1 microglia marker iNOS and CD86 were upregulated, but oppositely downregulated the level of M2 microglial markers (CD163 and Arginase-1) ($P < 0.05$ *vs* OE-NC group). In addition, in the si-Tug1 set, the p-p65/

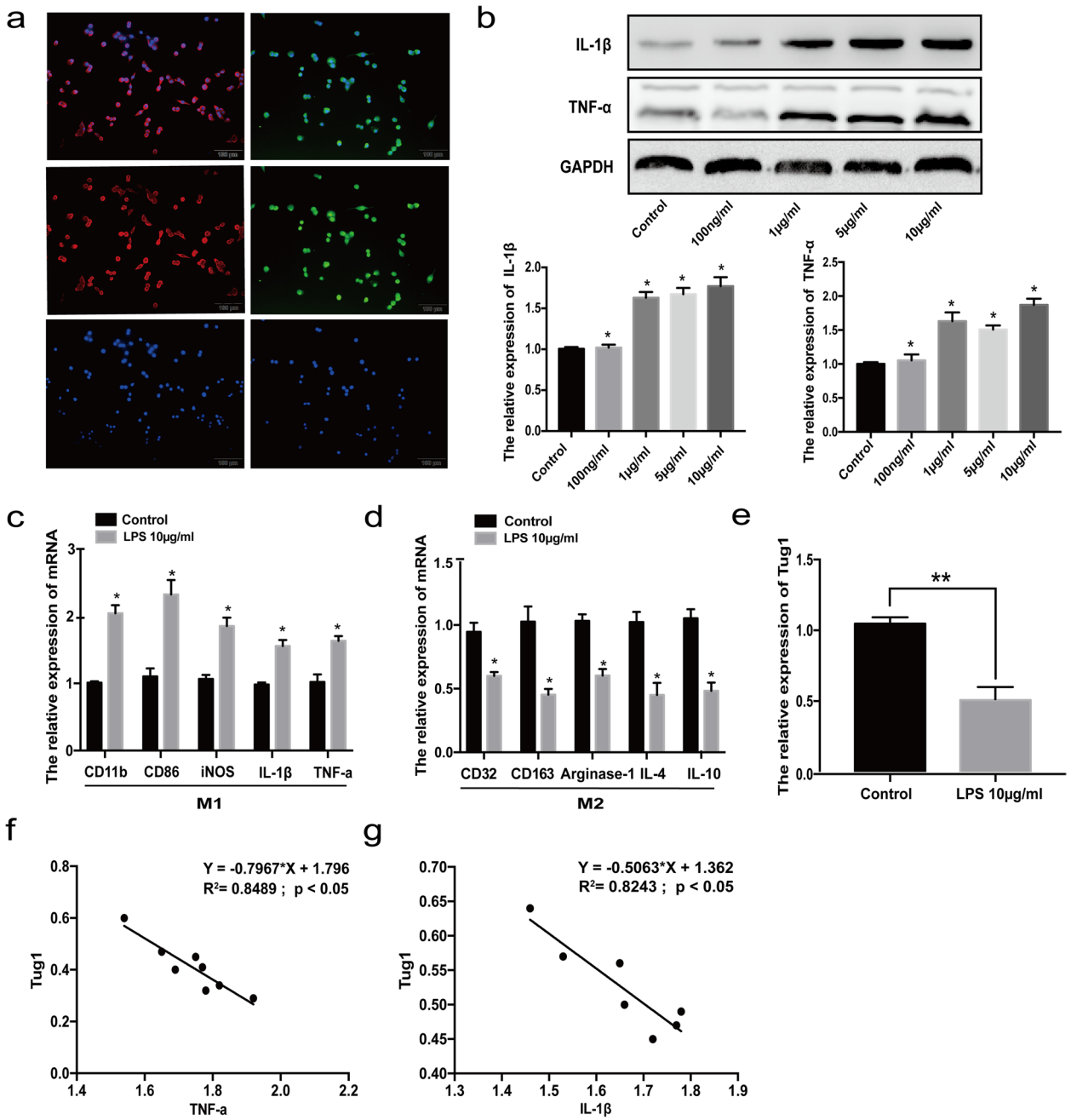

Fig. 3 Tug1 was downregulated in the LPS-treated microglia. **a** Phenotyping of microglial cells. The immunofluorescence showed positive expression of Iba1 and F4/80. **b** Western blotting analysis of IL-1β and TNF-α in rats at different LPS concentrations. **c** The mRNA expression of M1 type-associated markers CD11b, CD86, iNOS, IL-1β and TNF-α in microglial cells. **d** The mRNA expression of M2 type-associated markers CD32, CD163, Arginase-1, IL-4 and IL-10 in microglial cells. **e** The relative level of Tug1 in the LPS-treated microglia. **f-g** Pearson correlation analysis between Tug1 and TNF-α or IL-1β in the LPS-treated microglia. (*$P < 0.05$, **$P < 0.01$ vs Control)

p65 and p-IκBα/IκBα ratios were increased ($P < 0.05$ vs sh-NC group). The level of inflammatory factors (IL-1β and TNF-α) were also up-regulated in the si-Tug1 group ($P < 0.05$ *vs* control) in Fig. 5e-i. The observation described above suggest that Tug1 knockdown enhances the activation of the NF-κB signaling pathway, and promotes the expression of inflammatory molecular and M1 polarization of microglia.

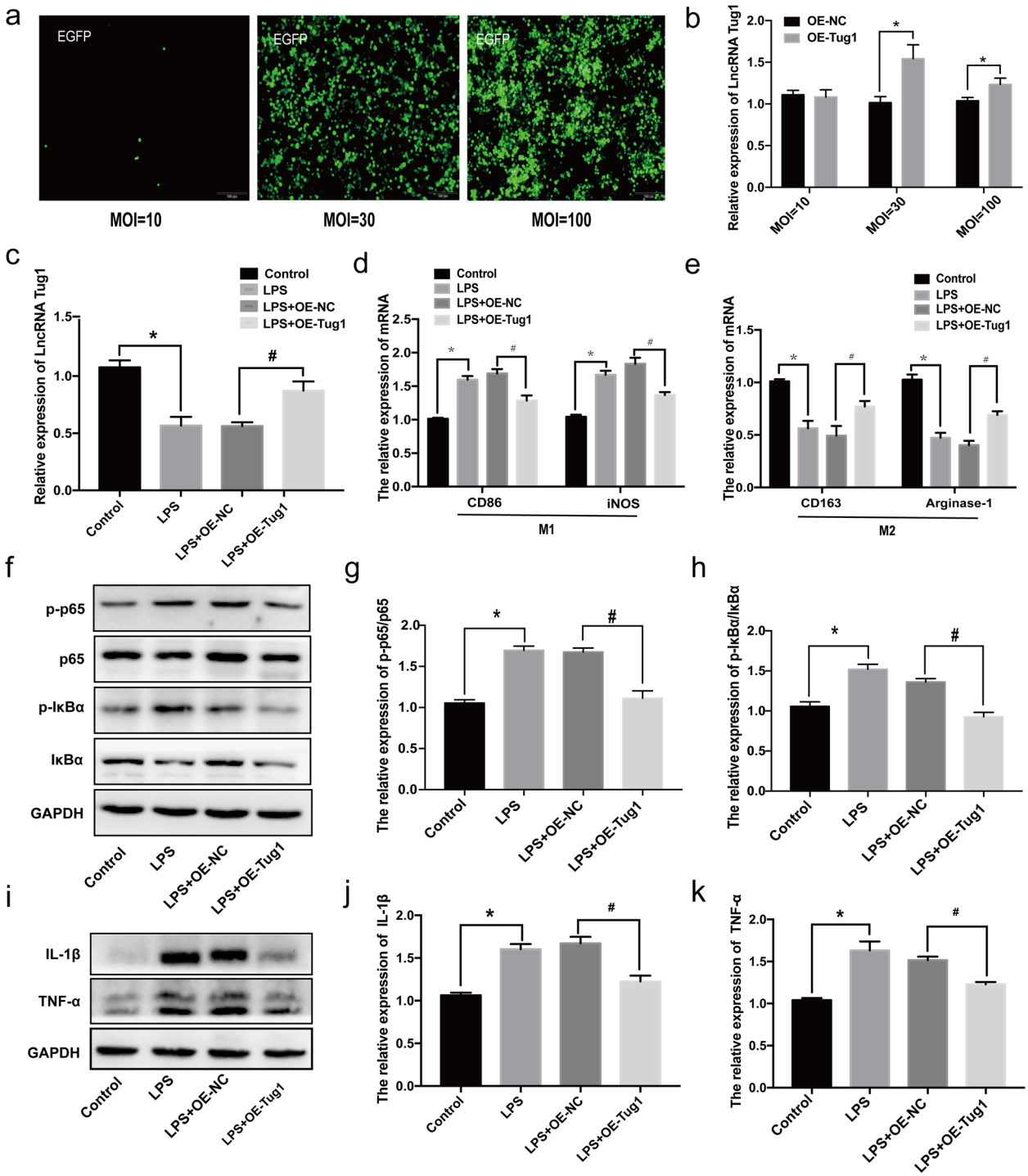

Fig. 4 Effect of Tug1 overexpression on LPS-induced inflammation in microglia. **a** Rat microglia infected with green fluorescence-labeled adenovirus. **b** Transfection efficiency of lncRNA Tug1 overexpression adenovirus at different multiplicities of infection. **c** Overexpression of lncRNA Tug1 in microglia. **d** The level of M1-associated markers mRNA in microglia cells. **e** The mRNA of M2-associated markers CD163 and Arginase-1 in rat microglia. **f** Protein bands of p-p65, p65, p-IκBα and IκBα in rat microglia. **g-h** The relative level of p-p65/p65 and p-IκBα/IκBα. **i** Protein bands of IL-1β and TNF-α in rat microglia. **j-k** The relative protein level of IL-1β and TNF-α. (*$P < 0.05$, **$P < 0.01$ vs. Control; #$P < 0.05$, ##$P < 0.01$ vs. LPS + OE-NC)

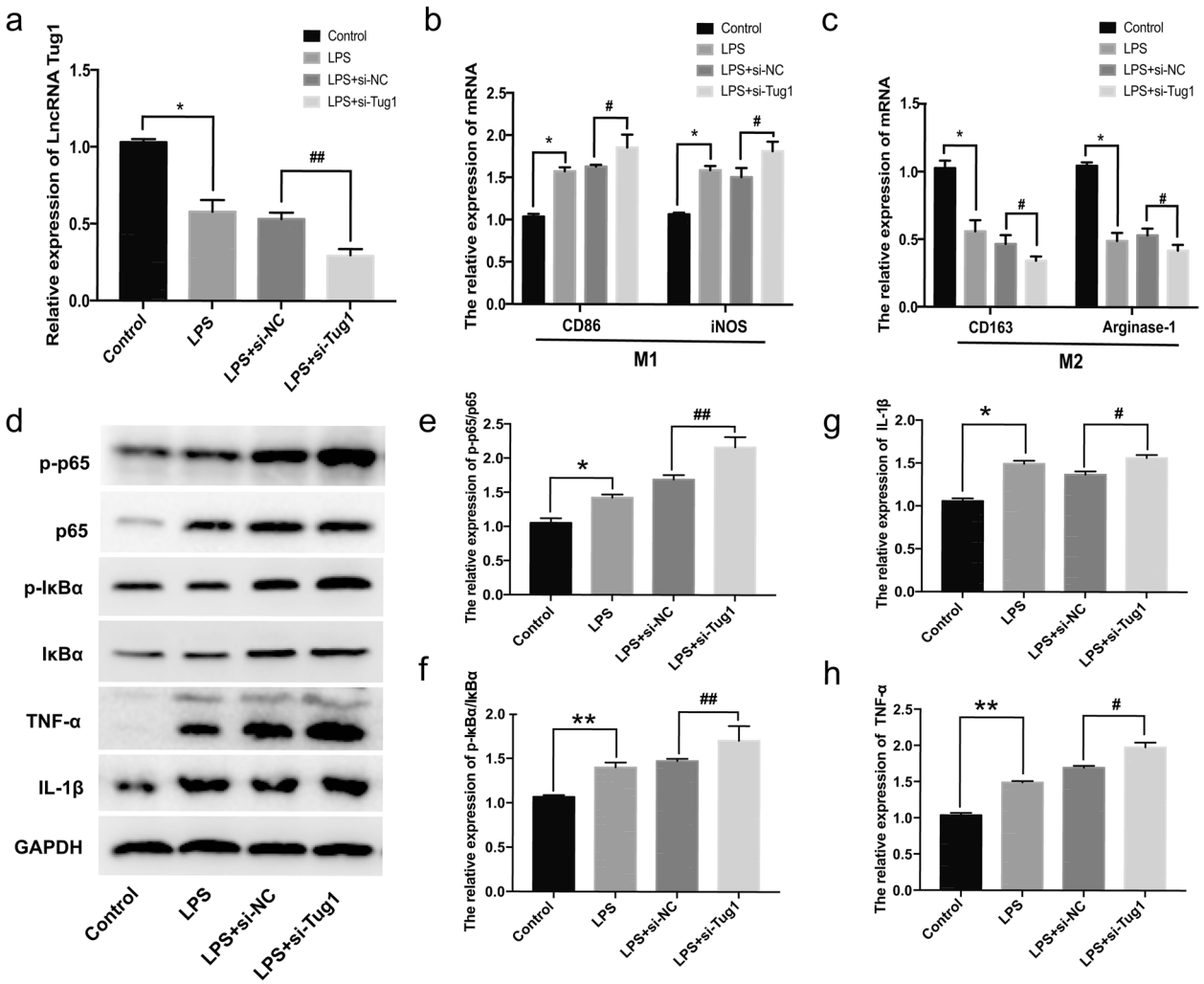

Fig. 5 Effect of Tug1 knockdown on LPS-treatment inflammation in microglia. **a** Knockdown of lncRNA Tug1 in microglia. **b** The mRNAlevel of M1-associated markers CD86 and iNOS in microglia. **c** The mRNA of M2-associated markers CD163 and Arginase-1 in rat microglia. **d** Western blotting analysis of p65, p-p65, pIκBα, IκBα, TNF-α and IL-1β in microglia. **e-h** The relative protein levels of p65, p-p65, pIκBα, IκBα, TNF-α and IL-1β in microglia. (*$P < 0.05$, **$P < 0.01$ vs. Control; #$P < 0.05$, ##$P < 0.01$ vs. LPS + si-NC)

Discussion

Neuroinflammation has been considered as an important factor for the development of SE[7, 15]. At the molecular level, moderate inflammatory responses promote tissue repair[16]. However, uncontrolled inflammation is involved in many pathological processes. In the brain, excessive inflammation shapes a neurotoxic environment that mediates neuronal dysfunction and cell death[9]. Thus, neuroinflammation can not only influence SE occurrence and development, but also change its prognosis. The NF-κB signaling pathway is considered to be tightly associated with inflammation in various types of cells[17]. As mentioned in previous studies[18], Tug1 is an important non-coding RNA that regulates gene expression and is involved in NF-κB pathway regulation[12, 19]. However, the mechanism by which Tug1 contributes to neuroinflammation after SE has been unclear.

In the current study, inflammation was induced by LPS in the RM. In the presence of LPS at 10 μg/ml, inflammation was apparent in microglia. Nonetheless, the in vitro condition cannot fully simulate the one in vivo. We consider that the inflammation in the microglia could partly mimic the neuroinflammation in vivo. The result of RIP in the present study predicted a binding site for Tug1 on P65, which provide precise

data for the mechanism of Tug1 in the NF-κB signaling pathway. Moreover, it provides the molecular basis for future studies on neuroinflammation after SE.

Furthermore, the level of Tug1 was decreased in the hippocampus of the rat SE model. This result was consistent with the expression of Tug1 in the LPS stimulated microglia. The expression of Tug1 was downregulated and negatively related with the expression of the inflammatory factors. Thus, Tug1 regulates inflammatory responses via the NF-κB signaling pathway in SE. Our further experiments showed that Tug1 down-regulation promoted inflammation by upregulating p-p65 and activated the NF-κB signal pathway. Moreover, up-regulated of Tug1 reduced the inflammation in LPS -treated microglia through inhibiting the expression of p-p65. These observations collectively indicate that Tug1 regulates inflammation in microglial via the NF-κB signaling pathway. This observation was in agreement with several previous research[20]. For example, Tug1 enhances insulin and alleviates inflammation in adipose tissues and alleviates the LPS-induced mouse hepatocyte inflammation[14, 21]. Therefore, Tug1 seems to be a potential target for inhibiting neuroinflammation.

Our results confirmed that lncRNA Tug1 controls inflammatory responses by directly binding to the NF-κB p65. However, for the mechanism of Tug1, there have been also different opinions in previously studies. Prior studies have shown that Tug1 inhibits inflammation by binding to PGC-1α protein in diabetic rats[22]. Similarly, Tug1 inhibits the LPS-induced inflammation in lung epithelial cells by targeting miR-34b-5p[23]. A possible explanation may be that Tug1 has multiple targets in different cells. The value of these difference suggests that the lncRNA Tug1 is an endogenous molecule that can effectively regulate inflammation.

This research extended our knowledge of the function of lncRNA Tug1 in inflammation. However, a number of limitations need to be considered. First, the inflammation in LPS-treated microglia was partially mimics the neuroinflammation in vivo. Second, the length of Tug1 was 4502 bp, which lack of overexpression vector in rat. The emergence of new technologies or new Tug1 carriers is important for eliminating the gap between basic experiments and clinical treatment. Therefore, further investigations are needed to make Tug1 a new therapy target for neuroinflammation.

Conclusions

In conclusion, our research showed that the lncRNA Tug1 inhibits inflammation in LPS-treated microglia through regulating the NF-κB signaling pathway. These findings will provide a better fundament for the molecular mechanisms that regulate the occurrence and development of neuroinflammation, which would provide foundations for the treatment of SE.

Availability data and materials

Not applicable

Abbreviations

SE: Status epilepticus; Tug1: Taurine up-regulated gene1; LPS: Lipopolysaccharide; RIP: RNA immunoprecipitation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42494-022-00080-6.

Additional file 1.

Additional file 2.

Additional file 3.

Additional file 4.

Authors' contribution

CYH and XYM designed the study. WM performed the experiments and write the manuscript. CYH and SYY review and edit the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Shanghai public clinical health center of Fudan University (No. 2019JS015).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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