Long Non-coding Antisense RNA DDIT4-AS1 Regulates Meningitic Escherichia coli-Induced Neuroinflammation by Promoting DDIT4 mRNA Stability

Bo Yang1,2 · Bojie Xu1,2 · Ruicheng Yang1,2 · Jiyang Fu1,2 · Liang Li1,2 · Dong Huo1,2 · Jiaqi Chen1,2 · Xiaopei Yang1,2 · Chen Tan1,2,3,4 · Huanchun Chen1,2,3,4 · Xiangru Wang1,2,3,4

Received: 19 July 2021 / Accepted: 8 December 2021 / Published online: 5 January 2022
© The Author(s) 2022

Abstract
Our previous studies have shown that meningitic Escherichia coli can colonize the brain and cause neuroinflammation. Controlling the balance of inflammatory responses in the host central nervous system is particularly vital. Emerging evidence has shown the important regulatory roles of long non-coding RNAs (lncRNAs) in a wide range of biological and pathological processes. However, whether lncRNAs participate in the regulation of meningitic E. coli-mediated neuroinflammation remains unknown. In the present study, we characterized a cytoplasm-enriched antisense lncRNA DDIT4-AS1, which showed similar concordant expression patterns with its parental mRNA DDIT4 upon E. coli infection. DDIT4-AS1 modulated DDIT4 expression at both mRNA and protein levels. Mechanistically, DDIT4-AS1 promoted the stability of DDIT4 mRNA through RNA duplex formation. DDIT4-AS1 knockdown and DDIT4 knockout both attenuated E. coli-induced NF-κB signaling as well as pro-inflammatory cytokines expression, and DDIT4-AS1 regulated the inflammatory response by targeting DDIT4. In summary, our results show that DDIT4-AS1 promotes E. coli-induced neuroinflammatory responses by enhancing the stability of DDIT4 mRNA through RNA duplex formation, providing potential nucleic acid targets for new therapeutic interventions in the treatment of bacterial meningitis.

Keywords Antisense lncRNA · DDIT4-AS1 · DDIT4 · Escherichia coli · Neuroinflammation

Introduction
Bacterial meningitis is the most important life-threatening infection of the central nervous system (CNS) and continues to be a significant cause of mortality and morbidity [1, 2].

Despite advances in antimicrobial treatment, survivors suffer from neurological sequelae including cognitive impairment, developmental delay, and hearing loss [1, 3]. Escherichia coli (E. coli) is the most common Gram-negative bacillary organism that causes meningitis in neonates and children, and hematogenous spread is the leading spreading way of E. coli meningitis [4, 5]. Our earlier studies have shown that meningitic E. coli can colonize the brain and cause neuroinflammation [6, 7]. However, how host respond to invading bacteria and modulate neuroinflammatory responses are still poorly understood.

In recent years, there has been increasing interest in long non-coding RNAs (lncRNAs). These are defined as RNAs longer than 200 nucleotides in length with no protein-coding capacity [8] and can be further classified as antisense lncRNAs, long intergenic noncoding RNAs (lincRNAs), intronic lncRNAs, and enhancer RNAs (eRNAs) based on their genome position [9]. Accumulating evidence has shown that lncRNAs play significant regulatory roles in diverse biological processes [10]. Further, they have been proposed to...
perform their functions through diverse mechanisms, including binding with RNA or DNA through nucleic acid base pairing, interacting with proteins through higher-order RNA structures [9, 11, 12]. However, our knowledge about the function and the potential molecular regulatory mechanisms of lncRNAs in bacterial meningitis is still limited.

DNA damage inducible transcript 4 (DDIT4), also known as REDD1/RTP801/Dig2, was originally characterized by its transcriptional upregulation in response to DNA damage. DDIT4 is an inhibitor of mammalian target of rapamycin (mTOR) and is induced by multiple cellular stresses including hypoxia, heat shock, energy depletion, starvation, and LPS [13, 14]. DDIT4 participates in regulating a broad spectrum of cellular and biological functions, such as cell survival, growth, apoptosis, and autophagy [15, 16]. Importantly, a growing body of evidence suggests that DDIT4 plays a crucial role in inflammation [14, 17–20]. Nevertheless, the function of DDIT4 in the CNS remains poorly characterized, especially in the context of bacterial infection.

In the present study, we characterized DDIT4-AS1, a long non-coding antisense transcript for DDIT4, as a cytoplasm-enriched antisense lncRNA and showed similar concordant expression patterns with DDIT4 upon E. coli infection. In addition, DDIT4-AS1 was found to positively regulate DDIT4 expression by promoting the stability of DDIT4 mRNA through RNA duplex formation. Decreasing the expression of DDIT4-AS1 or DDIT4 attenuated E. coli-induced pro-inflammatory factors production and NF-κB signaling. Moreover, we demonstrated that DDIT4-AS1 regulates the inflammatory response by targeting DDIT4. Taken together, these findings reveal that DDIT4-AS1 regulates meningitic E. coli-induced neuroinflammation by promoting DDIT4 mRNA stability, providing novel nucleic acid targets for future prevention and treatment of bacterial meningitis.

Materials and Methods

Bacterial Strains

The meningitic E. coli strain PCN033 used herein is a highly virulent cerebrospinal fluid isolate, originally isolated in Hunan Province, China, in 2006 [21]. The PCN033 strain was routinely grown aerobically at 37 °C in Luria–Bertani (LB) medium. The strain was evidenced to be capable of causing host blood–brain barrier (BBB) disruption and severe neuroinflammation in vitro and in vivo [6].

Cell Culture and Infection

The human astrocyte cell line U251 was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human brain microvascular endothelial cells (hBMECs) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, essential amino acids, nonessential amino acids, vitamins, penicillin, and streptomycin (100 U/mL). The human microglia cell line HMO6 was cultured in DMEM supplemented with 10% heat-inactivated FBS. All cells were incubated in a 37 °C incubator with a 5% CO2 atmosphere until monolayer confluence. Confluent cells were washed three times with phosphate-buffered saline (PBS) and starved in serum-free medium for 16–18 h prior to infection. Overnight cultures of PCN033 were resuspended and diluted in serum-free medium and then added to the starved monolayer cells at a multiplicity of infection (MOI) of 10.

Reagents

The DDIT4 (rabbit) antibody (#10,638–1-AP, 1:1000 dilution) and β-actin (mouse) antibody (#66,009–1-Ig, 1:5000 dilution) were obtained from Proteintech (Chicago, IL, USA). Anti-NF-kB/p65 (#6956, 1:1000 dilution) and anti-phospho-p65 (#3033, 1:1000 dilution) were obtained from Cell Signaling Technology (Danvers, MA, USA). Cy3-labeled goat anti-mouse (#A0521, 1:200 dilution) and FITC-labeled goat anti-rabbit antibodies (#A0562, 1:200 dilution) and DAPI were obtained from Beyotime Institute of Biotechnology (China). The DDIT4-AS1 antisense oligonucleotides (ASO) and the control ASO were purchased from Integrated Biotech Solutions Co., Ltd. (Shanghai, China); the sequences are listed in Table S2. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 plasmid pYSY-spCas9-sgRNA-Puro was obtained from YSY Biotech (Nanjing, China). The transfection reagent jetPRIME was purchased from Polyplus Transfection (Illkirch, France). The RNA polymerase II transcription inhibitor α-amanitin was purchased from Medchem Express (Princeton, NJ, USA). RNase A + T cocktail was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The super electrochemiluminescence (ECL) kit was obtained from US Everbright Inc. (Suzhou, China).

RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from astrocytes U251 cells using TRIzol® Reagent (Aidlab Biotech, Beijing, China) in accordance with the manufacturer’s protocol. One microgram of the total RNA was subjected to cDNA synthesis using HiScript II Q RT SuperMix (Vazyme, Nanjing, China). Real-time PCR was performed with the MonAmp™ SYBR Green qPCR Mix (RN040005M,Monad Biotech Co., Ltd, Wuhan, China) according to the manufacturer’s instructions. The transcriptional levels of all mRNA targets were
modified with a 5’, and each DNA oligonucleotide probe was
modified with a 5’ biotin. The sequences of the probes
are presented in Table S2. RAP was performed using the
RAP Kit (BersinBio, Guangzhou, China). According to the
manufacturer’s instruction, approximately 4 × 10⁷ cells were
crosslinked with 1% formaldehyde. The crosslinked cells were
solubilized using lysis buffer with protease inhibitor and
RNase inhibitor, followed by DNA elimination using
DNase. The solubilized lysates were subsequently incubated
with the prepared DDIT4-AS1 probe mixture or the control
probes and then immobilized with streptavidin-coated mag-
netic beads. Beads with captured hybrids were washed five
times with washing buffer. RNA elution buffer was added
to release the RNA from the beads and proteinase K was
used to remove all proteins. Finally, RNA was isolated and
subjected to qPCR assays.

**RNase Protection Assay**

The procedure was performed as previously described [23].
Two sets of primers were used. The first targeted the overlap-
ning (OL) region of DDIT4 and DDIT-AS1, and the second
targeted the non-OL region of DDIT4 mRNA. The RNA
samples from astrocytes were incubated at 37 °C for 1 h and
then treated with RNase A + T cocktail at 37 °C for 30 min.
The samples were then treated with proteinase K at 50 °C
for 1 h, followed by RNA purification using a MicroElute
RNA Clean-up Kit (Norcross, GA, USA). Subsequently, the
purified RNAs were subjected to cDNA synthesis and PCR
amplification to detect the OL and non-OL regions of DDIT4
mRNA, using two distinct sets of primers.

**α-Amanitin Treatment**

The astrocytes were transfected with the DDIT4-AS1 over-
expression plasmid or control plasmid. After 24 h, these
cells were treated with 5 μM α-amanitin for 6 h and then the
cells were harvested for RNA extraction and qPCR. Three
independent samples were obtained from each group.

**CRISPR/Cas9 Genomic Editing**

Two sgRNAs targeting human DDIT4 were cloned into the
all-in-one vector to generate the pYSY-spCas9-DDIT4-
sgRNA-Puro plasmids. Astrocytes were transfected with the
two CRISPR/Cas9 plasmids using jetPRIME. The cells were
incubated at 37 °C with 5% CO₂ for 24 h, and then, 400 ng/
ml puromycin was added and incubated for another 48 h.
The surviving cells were then transferred into 96-well plates
with limiting dilution and incubated at 37 °C with 5% CO₂
until a single-cell clone was formed. Genomic DNA from
each cell clone was extracted using the QuickExtract DNA
Extraction Solution (YSY Biotech, Nanjing, China). PCR
was performed to amplify the target region with the follow-
ing primers: 5′-CTTACAGCGGCTTCTACGC-3′ (forward)
and 5’-GGCTCTGACCCCTTCCAG-3’ (reverse). Finally, the positive editing cells were validated by sequencing.

Western Blotting

The astrocytes were lysed in radio immunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail (Sigma-Aldrich, USA) and then centrifuged at 12,000 rpm for 10 min at 4 °C. A BCA protein assay kit (Beyotime, China) was used to measure the protein concentration in the supernatant and the cell lysates were then subjected to western blot analyses as previously described [6]. The blots were visualized with ECL reagents.

Immunofluorescence Microscopy

The astrocytes were washed with PBS three times and fixed with 4% paraformaldehyde. The fixed cells were treated with 1% Triton X-100 in PBS and blocked in 5% BSA in Tris-buffered saline with Tween 20 (TBST), and then incubated with the primary antibody. Herein, DDIT4 was labeled with FITC and p65 was labeled with Cy3. The cells in the dishes were mounted and visualized under a fluorescence microscope.

Statistical Analysis

Data are expressed as the mean±SD and the significance of differences between groups was evaluated by unpaired two-tail t-test or one-way analysis of variance (ANOVA) embedded in GraphPad Prism, version 7.0 (GraphPad Software Inc., La Jolla, CA, USA). A level of \( p < 0.05 \) (*) was considered significant, and \( p < 0.01 \) (**) or \( p < 0.001 \) (***) was considered extremely significant.

Results

Antisense IncRNAs Display Differential Expression upon Meningitic E. coli Infection

We have previously performed IncRNAs sequencing in meningeal E. coli-infected astrocytes [24]. The expression profiling data revealed that 74 IncRNAs were differentially expressed, including 45 upregulated and 29 downregulated. In addition, the expression of 2045 mRNAs was significantly changed upon E. coli infection, of which 1150 were upregulated and 895 were downregulated. Subgroup analysis showed genomic classification of differentially expressed IncRNAs in Fig. 1a; lincRNAs represented the largest category (63.5%) of all differentially expressed IncRNAs, followed by antisense IncRNAs, which accounted for 28.4% (Table S4). A growing body of evidence suggests that antisense IncRNAs are frequently functional and regulate the expression of their sense protein-coding RNAs through diverse regulatory mechanisms, including transcription-related modulation, RNA–DNA interactions, and RNA–RNA interactions [25, 26]. To characterize the role of antisense IncRNAs in the pathological process of meningitic E. coli infection, we evaluated the expression of 21 antisense IncRNAs and their corresponding protein-coding mRNAs. We found that nine IncRNA–mRNA pairs were differentially expressed upon E. coli infection, and all of them showed concordant patterns of expression, including seven upregulated pairs (RP11-442H21.2/DDIT4, RP11-624G17.3/RTN4RL2, RP11-796E2.4/IRF1G1, RP11-809N8.2/RELT, RP4-781K5.2/IRF2BP2, AC093673.5/ZYX, and RP11-445F12.1/LHX1) and 2 downregulated pairs (CTD-2540B15.11/CEBPA and RP11-1143G9.4/LYZ) (Fig. 1b and c) (Table S5). Quantitative real-time PCR was performed for verification of differentially expressed IncRNA–mRNA pairs. As shown in Fig. 1d, six IncRNA–mRNA pairs displayed concordant expression, among which the RP11-442H21.2/DDIT4 pair showed the most significant difference. Combined with the fact that RP11-442H21.2 was highly expressed, as compared with other differentially expressed IncRNAs, and that DDIT4 has been reported to be involved in inflammation, the RP11-442H21.2/DDIT4 pair was chosen for further study.

DDIT4-AS1 Is a Cytoplasm-Enriched Antisense IncRNA

Based on the UCSC genome browser, RP11-442H21.2 is located at chromosomal band 10q22.1 and consists of two exons with a full length of 847 nt. RP11-442H21.2 was identified as a single antisense IncRNA transcribed from the reverse strand of the DDIT4 locus; therefore, it is also named DDIT4-AS1. The whole sequence of DDIT4-AS1 is shown in Table S3. As shown in Fig. 2a, the full length of DDIT4-AS1 shares a reverse complement region with the third exon of DDIT4, which is an 847 nt long sequence that we referred to as the overlapping (OL) region. To exclude the coding potential of DDIT4-AS1, three tools, including Coding-Non-Coding Index (CNCI) [27], Coding Potential Calculator (CPC) [28], and Predictor of Long Non-coding RNAs and Messenger RNAs Based on K-mer Scheme (PLEK) [29] were utilized to perform coding-potential analysis. Two well-known IncRNAs, XIST and HOTAIR, as well as three mRNAs, DDIT4, GAPDH, and β-actin, were used as controls. It is apparent from Fig. 2b that DDIT4-AS1 had a very low coding potential and comparable to XIST and HOTAIR. In addition, we predicted a short 141-nt small ORF in DDIT4-AS1 with the potential to encode peptide; the sequence was shown in Table S3. A series of constructs were generated to further validate the coding
potential of DDIT4-AS1 (Fig. S1a). pEGFP-N1-MUT was generated by mutating the start codon ATG to ATT in pEGFP-N1 vector. The DDIT4-AS1 ORF sequence and DDIT4 CDS sequence were cloned into the pEGFP-N1-MUT. As expected, substantial expression of the EGFP was observed in pEGFP-N1-WT-transfected cells, while mutation of the start codon abolished the expression of the EGFP protein. Importantly, the expression of the EGFP was observed in DDIT4-pEGFP-N1-MUT-transfected cells, but not in DDIT4-AS1-ORF-pEGFP-N1-MUT-transfected cells, which further proved the non-coding feature of DDIT4-AS1 (Fig. S1b). In order to investigate the subcellular localization of DDIT4-AS1, a FISH assay was conducted, and the results showed that DDIT4-AS1 was enriched in the cytoplasm of astrocytes (Fig. 2c). This result was further confirmed by the quantification of nucleus/cytoplasm RNAs (Fig. 2d). Collectively, these data indicate that DDIT4-AS1 is a cytoplasm-enriched antisense lncRNA.

DDIT4-AS1 and DDIT4 Are Upregulated upon Meningitic E. coli Infection

We next examined the expression of DDIT4-AS1 and DDIT4 upon meningitic E. coli infection. To prevent non-specific amplification, the forward primer of DDIT4-AS1 was designed to span the first and second exons. The primers of DDIT4 were designed on the second exon to distinguish DDIT4-AS1 and DDIT4. We first analyzed the level of DDIT4-AS1 in the E. coli-infected astrocyte cell line U251 using qPCR. The results showed that the expression...
of DDIT4-AS1 was significantly increased in a dose- and time-dependent manner (Fig. 3a, b). In addition, the mRNA and protein levels of DDIT4 also elevated in a time-dependent manner (Fig. 3c, d). Furthermore, we detected the expression of DDIT4-AS1 and DDIT4 in human brain microvascular endothelial cells (hBMECs) and microglia cell line HMO6. The results were similar to those observed in E. coli-infected astrocytes; E. coli infection induced the notable upregulation of DDIT4-AS1 and DDIT4 in hBMECs (Fig. 3e–h) and microglia (Fig. 3i–l). The similar concordant expression patterns of DDIT4-AS1 and DDIT4 indicated a strong correlation between them. Given that astrocytes and hBMECs are the two major BBB cell types and microglia are considered the major inflammatory cell type in the CNS, the DDIT4-AS1/DDIT4 pair might play an important role in the CNS upon E. coli infection.
DDIT4-AS1 Positively Regulates DDIT4 Expression

To determine whether DDIT4-AS1 can regulate the expression of DDIT4, we transiently downregulated it in astrocytes using modified ASO, which is a single-strand RNA targeting DDIT4-AS1 without directly affecting the expression of DDIT4. DDIT4-AS1-depleted cells showed decreased DDIT4 mRNA levels (Fig. 4a). In addition, western blot and immunofluorescence results showed that DDIT4 protein levels were also reduced in DDIT4-AS1-depleted cells (Fig. 4b, c). We also overexpressed DDIT4-AS1 by transfecting the full-length sequence of DDIT4-AS1 in astrocytes; as we expected, overexpression of DDIT4-AS1 induced increased mRNA and protein expression of DDIT4 (Fig. 4d–f). These findings indicate that DDIT4-AS1 positively regulates DDIT4 expression.
DDIT4-AS1 Increases DDIT4 mRNA Stability by Forming an RNA Duplex

Based on the reverse complement nucleotides between DDIT4-AS1 and DDIT4, we speculated that DDIT4-AS1 and DDIT4 could form an RNA duplex to increase the stability of DDIT4 mRNA. To test our hypothesis, we first examined the cellular localization of DDIT4-AS1 and DDIT4 by FISH. As shown in Fig. 5a, some fluorescence signals of DDIT4-AS1 and DDIT4 overlapped, implying that DDIT4-AS1 is likely to interact with DDIT4 in the cytoplasm. To further verify the direct interaction between DDIT4-AS1 and DDIT4, RNA antisense purification (RAP) was conducted using biotin-labeled RNA probes targeting DDIT4-AS1. We observed that DDIT4 mRNA was significantly enriched in biotin-labeled DDIT4-AS1 pull-down samples compared to livers in negative control (Fig. 5b). In addition, we used an RNase protection assay (RPA) on RNA from astrocytes to confirm the formation of the RNA duplex. The detection probes were designed on the overlapping (OL) and non-OL regions of DDIT4. PCR amplification results showed that the non-OL region was completely digested by RNase, whereas the OL region was partially protected from degradation (Fig. 5c). We next evaluated the effect of DDIT4-AS1 on the stability of DDIT4 by blocking new RNA synthesis with the RNA polymerase II transcription inhibitor α-amanitin over a 6-h period. As shown in Fig. 5d, 18 s ribosomal RNA, a product of RNA polymerase I, showed no significant changes upon α-amanitin treatment. Approximately 60% of DDIT4 mRNA was consumed after 6 h, whereas the expression of DDIT4 was partially restored by overexpressing DDIT4-AS1, which revealed that the stability of DDIT4 was elevated by DDIT4-AS1. Taken together, these data demonstrate that DDIT4-AS1 and DDIT4 could form an RNA duplex to increase DDIT4 mRNA stability.

Knockdown of DDIT4-AS1 Suppresses E. coli-Induced Pro-inflammatory Factors Production and NF-κB Signaling

Considering that DDIT4 has been implicated in the regulation of inflammatory responses and DDIT4-AS1 can positively regulate DDIT4 expression, we next examined the effect of DDIT4-AS1 knockdown on pro-inflammatory gene expression in astrocytes. As shown in Fig. 6a, DDIT4-AS1 knockdown significantly inhibited E. coli-induced pro-inflammatory gene expression, including IL-1β and TNF-α. NF-κB-mediated pro-inflammatory gene expression plays a crucial role in the innate immune response against bacterial infection; thus, we evaluated the effect of DDIT4-AS1 on NF-κB signaling.
Fig. 5  

**a** Representative images of RNA FISH showing co-localization of *DDIT4-AS1* (red) with *DDIT4* mRNA (green) in the cytoplasm. The cell nucleus was stained in blue with DAPI. Scale bar indicates 20 μm. **b** qPCR was used to measure the enrichment of *DDIT4* mRNA in biotin-labeled *DDIT4-AS1* pull-down samples compared to that in the negative control. **c** RNase protection assay was performed on RNA samples from U251 cells, and PCR amplification was used to detect the overlapping (OL) and the non-OL regions of *DDIT4*. **d** U251 cells were transfected with the *DDIT4-AS1* overexpression plasmid or the empty vector for 24 h and then treated with 5 μM α-amanitin for 6 h. The expression of 18S and *DDIT4* was determined by qPCR. Error bars in **b** and **d** represent the mean ± SD (*n* = 3/group). *p* < 0.05 (*), *p* < 0.001 (***).
knockdown on NF-κB signaling. *E. coli* infection stimulated the phosphorylation of p65, and this effect was attenuated by the knockdown of *DDIT4-AS1* (Fig. 6b). We further examined the effect of *DDIT4-AS1* on NF-κB signaling using immunofluorescence microscopy. As expected, *E. coli* infection promoted p65 translocation from the cytoplasm to the nucleus, which was partly prevented by *DDIT4-AS1* knockdown (Fig. 6c). Collectively, these results suggest that *DDIT4-AS1* regulates pro-inflammatory factors production and NF-κB signaling.

**DDIT4-AS1 Regulates the Inflammatory Response by Targeting DDIT4**

To further verify that *DDIT4-AS1* regulates the inflammatory response by targeting *DDIT4*, we evaluated the function of *DDIT4* through deletion using the CRISPR/Cas9 approach. Two small guide RNAs were designed to target exon 2 of *DDIT4*, and the deletion was validated by PCR amplification (Fig. 7a). In addition, *E. coli* infection led to the upregulation of *DDIT4*, which was abolished by *DDIT4*
deletion, with no DDIT4 expression in the knockout (KO) cells (Fig. 7b). DDIT4 knockout markedly suppressed E. coli-induced pro-inflammatory IL-1β and TNF-α expression, as well as infection-induced NF-κB p65 phosphorylation and nuclear translocation (Fig. 7c–e). Moreover, the overexpression of DDIT4-AS1 augmented E. coli-induced IL-1β and TNF-α expression; however, the pro-inflammatory effect of DDIT4-AS1 vanished in DDIT4 KO cells (Fig. 7f), which revealed that DDIT4-AS1 functions are mediated by DDIT4. Collectively, these data indicate that DDIT4-AS1 plays a pro-inflammatory role in the progress of E. coli infection by promoting DDIT4 mRNA stability.

Discussion

A growing body of evidence suggests that lncRNAs are involved in a wide range of biological functions and lncRNAs are now emerging as important regulators of inflammation [30]. Antisense lncRNAs are defined as long non-coding RNAs from the opposite strand of the sense transcript of either protein-coding or nonprotein-coding genes [26, 31]. Antisense lncRNAs have been reported to modulate almost every level of gene regulation, including pre-transcriptional, transcriptional, and post-transcriptional gene regulatory mechanisms, to exert a broad spectrum of biological functions [25]. Importantly, antisense lncRNAs can act as positive and negative regulators of the corresponding sense transcript [31–33]. In the current study, we sought to explore the involvement of lncRNAs in the regulation of meningitic E. coli-mediated neuroinflammation. We focused our attention on antisense lncRNAs and selected the most significantly upregulated lncRNA–mRNA pair (DDIT4-AS1/DDIT4) for further studies. We found that the cytoplasm-enriched antisense lncRNA DDIT4-AS1 showed concordant expression patterns with DDIT4 upon E. coli infection, and DDIT4-AS1 modulated DDIT4 expression by enhancing the stability of DDIT4 mRNA through RNA duplex formation, thereby promoting NF-κB activation and pro-inflammatory gene expression.

Meningitic E. coli infection of the host CNS relies on intricate interactions between the host BBB and bacteria. Our previous studies have characterized the transcriptome profiles of astrocytes in response to infection and revealed that lncRNAs are likely involved in the development of bacterial meningitis [24]. Indeed, the role of lncRNAs in the host cell response to bacterial infections has received increased attention in recent years, and studies show that lncRNAs actively respond to various bacterial infections, including Salmonella, Helicobacter pylori, Mycobacterium, and Listeria monocytogenes [34–38]. Interestingly, many lncRNAs have been reported to modulate inflammatory responses in the progress of bacterial infection. For example, an intergenic lncRNA lincRNA-EPS is downregulated in macrophages exposed to L. monocytogenes infection, which acts as a repressor of inflammatory responses by interacting with heterogeneous nuclear ribonucleoprotein L [39]. In contrast, the antisense lncRNA AS-ILL1a, which is partially complementary to IL-1α, is upregulated following L. monocytogenes infection. AS-ILL1a recruits RNA polymerase II to the IL-1α promoter, thereby enhancing IL1α expression [38]. In this study, we identified an E. coli infection–induced antisense lncRNA DDIT4-AS1 that could promote NF-κB signaling by upregulating DDIT4 expression. Our study further confirmed the important regulatory role of lncRNAs in the process of bacterial infection, which might provide potential new targets for future prevention of pathogenic E. coli meningitis.

We observed that DDIT4-AS1 and DDIT4 showed concordant expression patterns upon E. coli infection and DDIT4-AS1 positively modulated DDIT4 expression. Mechanistically, DDIT4-AS1 formed an RNA duplex with DDIT4 mRNA and enhanced its stability. Our explanation is that mRNA undergoes endonucleolytic or exonucleolytic degradation by various RNases; however, the RNA duplex formation could protect mRNAs from RNases degradation, thereby promoting mRNA stability [40]. A growing body of evidence suggests that antisense lncRNAs play important regulatory roles by forming RNA duplexes with mRNAs [23, 41–46]. A well-known case is BACE-AS1, which is transcribed from the opposite strand of BACE1. BACE-AS1 forms an RNA duplex with BACE1 and drives rapid feed-forward regulation of β-secretase [23]. In gastric cancer (GC), lncRNA KRT7-AS shows concordant expression with KRT7 in GC tissues and cell lines. KRT7-AS increases KRT7 mRNA stability by forming an RNA duplex, which promotes GC cell proliferation and migration [41]. In lung adenocarcinoma, lncRNA MUC5B-AS1 promotes cell migration and invasion by increasing the stability of MUC5B mRNA through a mechanism involving RNA duplex formation [42]. In addition, the formation of an RNA duplex by antisense lncRNA and mRNA might cover microRNA binding sites of the mRNA, thereby stabilizing the mRNA [47, 48], which generally occurs in cytoplasm-enriched lncRNAs. Nucleus-enriched antisense lncRNAs can promote mRNA stability by modulating the association of RNA-binding proteins [43, 44]. For example, antisense lncRNA Safe could form an RNA duplex with Sfrp2, and the RNA binding protein HuR could bind to the Safe–Sfrp2 RNA duplex and stabilize both Safe and Sfrp2 [44]. Our RNA FISH assays demonstrated that DDIT4-AS1 was located in cytoplasm and DDIT4-AS1 co-located with DDIT4. Further experiments showed that DDIT4-AS1 directly interacted with DDIT4 mRNA and protected it from degradation. Moreover, the overexpression of DDIT4-AS1 promoted the stability of DDIT4 mRNA after treatment with α-amanitin. Our studies, in conjunction with
**Fig. 1.** (a) Schematic diagram of DDIT4 region. The 5' and 3' regions are represented by E1 and E3, respectively. The sgRNA1 insertion site is marked by the red triangle. (b) Western blot analysis of DDIT4 protein expression in WT and DDIT4-KO mice under E. coli infection. (c) Expression levels of IL1β and TNF-α in WT and DDIT4-KO mice under E. coli infection. (d) Western blot analysis of p65 and β-actin protein expression in WT and DDIT4-KO mice under E. coli infection. (e) Immunofluorescence images of p65 and DAPI staining in WT and DDIT4-KO mice under E. coli infection. (f) Expression levels of IL1β and TNF-α in WT and DDIT4-KO mice under E. coli infection after transfection of pcDNA3.1 and pcDNA3.1-DDIT4-AS1.
Fig. 7. DDIT4-AS1 regulates inflammatory response by targeting DDIT4. a Two small guide RNAs (sgRNA1 and sgRNA2) were designed to target the second exon of DDIT4, and PCR was used to identify the DDIT4 knockout (KO) using specific primers (F and R). b The DDIT4-KO cells and the wild-type (WT) cells were infected with E. coli at an MOI of 10 for 3 h; the expression of DDIT4 was measured by western blotting. c qPCR was used to measure the expression of IL-1β and TNF-α in E. coli-infected DDIT4-KO cells and WT cells. d Western blot analysis of p65 phosphorylation in DDIT4-KO cells and WT cells upon E. coli infection. e Translocation of the p65 subunit was detected in DDIT4-KO cells and WT cells. The p65 was labeled in red, and the cell nucleus was stained in blue with DAPI. Scale bar: 50 μm. f The DDIT4-KO cells and WT cells were transfected with the DDIT4-AS1 overexpression plasmid and then infected with E. coli for 3 h, and the expression of IL-1β and TNF-α was determined by qPCR. GAPDH was used as the internal reference. Data represented the mean ± SD (n = 3/group). Statistical analysis was carried out by one-way ANOVA. p < 0.05 (*), p < 0.01 (**), p < 0.001 (***).

these previous findings, suggest that antisense lncRNAs can modulate their sense mRNAs by forming RNA duplexes. DDIT4 has emerged as an important regulator of inflammatory responses. In a mouse model of cigarette smoke, DDIT4 is determined to be upregulated in the lungs and forcefully expressing DDIT4 promotes NF-kB activation and further exacerbates alveolar inflammation. However, alveolar inflammation and lung injury are markedly abrogated in DDIT4 knockout mice [17]. DDIT4 also aggravates LPS-induced systemic inflammation in macrophages, and the inflammatory responses are attenuated by DDIT4 knockdown and knockout [14, 19]. In agreement with previous studies, the absence of DDIT4 in astrocytes also decreased E. coli-induced inflammation, which further verified the regulatory function of DDIT4 in inflammation. DDIT4 knock-out markedly alleviated the production of pro-inflammatory cytokines through the NF-kB signaling pathway. Recent evidence suggests that DDIT4 interacts with and sequesters IκBα, thus promoting IKK independent atypical NF-kB activation [14]. It is possible that DDIT4 knockout abrogated the sequestration of IκBα, which led to the suppression of NF-kB signaling. However, the specific molecular mechanisms require further investigation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12355-021-02690-6.

Author Contribution BY, XW, and HC conceived and designed the project. BY, BX, RY, and LL performed the experiments. BY, JF, DH, JC, and XY analyzed the data. XW, HC, and CT provide technical and administrative support. BY and XW drafted and revised the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by grants from the National Natural Science Foundation of China (31772736 and 32122086), the National Key R&D Program of China (2021YFD1800800), the National Natural Science Foundation of China (NSFC) (32102749), and the China Postdoctoral Science Foundation (2020M672379 and 2021T140242).

Data Availability All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate
Not applicable.

Consent for Publication
Not applicable.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Kim KS (2010) Acute bacterial meningitis in infants and children. Lancet Infect Dis 10(1):32–42. https://doi.org/10.1016/S1473-3099(09)70306-8
2. Wang X, Maruvada R, Morris AJ, Liu JO, Wolfgang MJ, Baek DJ, Bittman R, Kim KS (2016) Sphingosine 1-phosphate activation of EGFR as a novel target for meningitic Escherichia coli penetration of the blood-brain barrier. PLoS Pathog 12(10):e1005926. https://doi.org/10.1371/journal.ppat.1005926
3. Kim KS (2003) Pathogenesis of bacterial meningitis: from bacteriaemia to neuronal injury. Nat Rev Neurosci 4(5):376–385. https://doi.org/10.1038/nrn1103
4. Kim KS, Itabashi H, Genski P, Sadoff J, Warren RL, Cross AS (1992) The K1 capsule is the critical determinant in the development of Escherichia coli meningitis in the rat. J Clin Invest 90(3):987–905. https://doi.org/10.1172/JCI115965
5. Zhao WD, Liu DX, Wei JY, Miao ZW, Zhang K, Su ZK, Zhang XW, Li Q, Fang WG, Qin XX, Shang DS, Li B, Li QC, Cao L, Kim KS, Chen YH (2018) Caspr1 is a host receptor for meningitic Escherichia coli. Nat Commun 9(1):2296. https://doi.org/10.1038/s41467-018-04637-3
6. Yang R, Liu W, Miao L, Yang X, Fu J, Dou B, Cai A, Zong X, Tan C, Chen H, Wang X (2016) Induction of VEGFA and Snail-1 by meningitic Escherichia coli mediates disruption of the blood-brain barrier. Oncotarget 7(39):63839–63855. https://doi.org/10.18632/oncotarget.11696
7. Yang B, Yang R, Xu B, Fu J, Qu X, Li L, Dai M, Tan C, Chen H, Wang X (2021) miR-155 and miR-146a collectively regulate meningitic Escherichia coli infection-mediated neuroinflammatory responses. J Neuroinflammation 18(1):114. https://doi.org/10.1186/s12974-021-02165-4
8. Ponting CP, Oliver PL, Reik W (2009) Evolution and functions of long non-coding RNAs. Cell 136(4):629–641. https://doi.org/10.1016/j.cell.2009.02.006
9. Chen YG, Satapathy AT, Chang HY (2017) Gene regulation in the immune system by long noncoding RNAs. Nat Immunol 18(9):962–972. https://doi.org/10.1038/ni.3771
10. Beermann J, Piccoli MT, Viercke J, Thum T (2016) Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. Physiol Rev 96(4):1297–1325. https://doi.org/10.1152/physrev.00041.2015
11. Kopp F, Mendell JT (2018) Functional classification and experimental dissection of long noncoding RNAs. Cell 172(3):393–407. https://doi.org/10.1016/j.cell.2018.01.011
12. Long Y, Wang X, Youmans DT, Cech TR (2017) How do IncRNAs regulate transcription? Sci Adv 3(9):eaao2110. https://doi.org/10.1126/sciadv.aao2110
13. Tirado-Hurtado I, Fajardo W, Pinto JA (2018) DNA damage inducible transcript 4 gene: the switch of the metabolism as potential target in cancer. Front Oncol 8:106. https://doi.org/10.3389/fonc.2018.00106
14. Lee DK, Kim JH, Kim J, Choi S, Park M, Park W, Kim S, Lee KS, Kim T, Jung J, Choi YK, Ha KS, Won MH, Billiar TR, Kwon YG, Kim YM (2018) REDD1 aggravates endotoxin-induced inflammation via atypical NF-kappaB activation. FASEB J 32(8):4585–4599. https://doi.org/10.1096/fj.201701436R
15. Li B, Chen R, Chen L, Qiu F, Ai X, Huang E, Huang W, Chen C, Liu C, Lin Z, Xie WB, Wang H (2017) Effects of DDIT4 in methamphetamine-induced autophagy and apoptosis in dopaminergic neurons. Mol Neurobiol 54(3):1642–1660. https://doi.org/10.1007/s12035-016-9637-9
16. Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, Witters LA, Ellisen LW, Kaelin WG Jr (2004) Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev 18(23):2893–2904. https://doi.org/10.1101/gad.125680.4
17. Yoshida T, Mett I, Bhunia AK, Bowman J, Perez M, Zhang L, Gandjeva A, Zhen L, Chukwueke U, Mao T, Richter A, Brown E, Sanders LA, Gandjeva A, Chabon J, Koyanagi DE, Graham BB, Sussan T, Cosgrove G, Mouded M, Shapiro SD, Petrache I, Morgan TE, Finch CE, St-Laurent G 3rd, Kenny PJ, Wahlestedt C (2008) Expression of a noncoding RNA is elevated in Alzheimer’s disease and drives rapid feed-forward regulation of beta-secretase. Nat Med 14(7):723–730. https://doi.org/10.1038/nm1784
18. Yang B, Yin F, Yang R, Xu B, Fu J, Zhi S, Dai M, Tan C, Chen H, Wang X (2020) Holistic insights into meningitic Escherichia coli infection of astrocytes based on whole transcriptome profiling. Epigenomics 12(18):1611–1632. https://doi.org/10.2217/epi-2019-0342
19. Faghihi MA, Wahlestedt C (2009) Regulatory roles of natural antisense transcripts. Nat Rev Mol Cell Biol 10(9):637–643. https://doi.org/10.1038/nrm2738
20. Pelechano V, Steinmetz LM (2013) Gene regulation by antisense transcription. Rev Transl Genomics 4(12):880–893. https://doi.org/10.1038/ntg.2013.13
21. Chung JH, Lim SH, Seol HW, Lee DK, Kim JH, Kim J, Choi S, Park M, Park W, Kim S, Lee KS, Kim T, Jung J, Choi YK, Ha KS, Won MH, Billiar TR, Kwon YG, Kim YM (2018) REDD1 aggravates endotoxin-induced inflammation via atypical NF-kappaB activation. FASEB J 32(8):4585–4599. https://doi.org/10.1096/fj.201701436R
22. Castanotto D, Lingeman R, Riggins AD, Rossi JJ (2009) CRM1 mediates nuclear-cytoplasmic shuttling of mature microRNAs. Proc Natl Acad Sci U S A 106(51):21655–21659. https://doi.org/10.1073/pnas.0912384106
23. Faghihi MA, Modarresi F, Khalil AM, Wood DE, Sahagan BG, Morgan TE, Finch CE, St-Laurent G 3rd, Kenny PJ, Wahlestedt C (2008) Expression of a noncoding RNA is elevated in Alzheimer’s disease and drives rapid feed-forward regulation of beta-secretase. Nat Med 14(7):723–730. https://doi.org/10.1038/nm1784
24. Yang B, Yin F, Yang R, Xu B, Fu J, Zhi S, Dai M, Tan C, Chen H, Wang X (2020) Holistic insights into meningitic Escherichia coli infection of astrocytes based on whole transcriptome profiling. Epigenomics 12(18):1611–1632. https://doi.org/10.2217/epi-2019-0342
25. Faghihi MA, Wahlestedt C (2009) Regulatory roles of natural anti-sense transcripts. Nat Rev Mol Cell Biol 10(9):637–643. https://doi.org/10.1038/nrm2738
26. Pelechano V, Steinmetz LM (2013) Gene regulation by antisense transcription. Rev Transl Genomics 4(12):880–893. https://doi.org/10.1038/ntg.2013.13
27. Chung JH, Lim SH, Seol HW, Lee DK, Kim JH, Kim J, Choi S, Park M, Park W, Kim S, Lee KS, Kim T, Jung J, Choi YK, Ha KS, Won MH, Billiar TR, Kwon YG, Kim YM (2018) REDD1 aggravates endotoxin-induced inflammation via atypical NF-kappaB activation. FASEB J 32(8):4585–4599. https://doi.org/10.1096/fj.201701436R
28. Kong L, Zhang Y, Ye ZQ, Liu QX, Zhao SQ, Wei L, Gao G (2007) CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic Acids Res 35:W345-349. https://doi.org/10.1093/nar/gkm391
29. Li A, Zhang J, Zhou Z (2014) PLEK: a tool for predicting long non-coding RNAs and messenger RNAs based on an improved k-mer scheme. BMC Bioinformatics 15:311. https://doi.org/10.1186/1475-2877-15-311
30. Atianand MK, Caffrey DR, Fitzgerald KA (2017) Immunobiology of long noncoding RNAs. Annu Rev Immunol 35:177–198. https://doi.org/10.1146/annurev-immunol-041015-055459
31. Villegas VE, Zaphiropoulos PG (2015) Neighboring gene regulation by antisense long non-coding RNAs. Int J Mol Sci 16(2):3251–3266. https://doi.org/10.3390/ijms16023251
32. Wahlestedt C (2006) Natural antisense and noncoding RNA transcripts as potential drug targets. Drug Discov Today 11(11–12):503–508. https://doi.org/10.1016/j.drudis.2006.04.013
33. Meng W, Cui W, Zhao L, Chi W, Cao H, Wang B (2019) Aberrant methylation and downregulation of ZNF667-AS1 and ZNF667 promote the malignant progression of laryngeal squamous cell carcinoma. J Biomed Sci 26(1):13. https://doi.org/10.1186/s12028-019-0506-0
34. Gomez JA, Wapinski OL, Yang YW, Bureau JF, Gopinath S, Monack DM, Chang HY, Brahic M, Kirkegaard K (2013) The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-gamma locus. Cell 152(4):743–754. https://doi.org/10.1016/j.cell.2013.01.015
35. Zhu H, Wang Q, Yao Y, Fang J, Sun F, Ni Y, Shen Y, Wang H, Shao S (2015) Microarray analysis of long non-coding RNA expression profiles in human gastric cells and tissues with Helicobacter pylori infection. BMC Med Genomics 8:84. https://doi.org/10.1186/s12920-015-0159-0
36. Wang Y, Zhong H, Xie X, Chen CY, Huang D, Shen L, Zhang H, Chen ZW, Zheng G (2015) Long noncoding RNA derived from CD244 signaling epigenetically controls CD8+ T-cell immune responses in tuberculosis infection. Proc Natl Acad Sci U S A 112(29):E3883-3892. https://doi.org/10.1073/pnas.1501662112
37. Yi Z, Li J, Gao K, Fu Y (2014) Identification of differentially expressed long non-coding RNAs in CD4+ T cells response to latent tuberculosis infection. J Infect 69(6):558–568. https://doi.org/10.1016/j.jinf.2014.06.016
38. Chan J, Atianand M, Jiang Z, Carpenter S, Aiello D, Elling R, Fitzgerald KA, Caffrey DR (2015) Cutting edge: a natural antisense transcript, AS-IL1alpha, controls inducible transcription of the proinflammatory cytokine IL-1alpha. J Immunol 195(4):1359–1363. https://doi.org/10.4049/jimmunol.1500264

39. Atianand MK, Hu W, Satpathy AT, Shen Y, Ricci EP, Alvarez-Dominguez JR, Bhatta A, Schattgen SA, McGowan JD, Blin J, Braun JE, Gandhi P, Moore MJ, Chang HY, Lodish HF, Caffrey DR, Fitzgerald KA (2016) A long noncoding RNA lincRNA-EPS acts as a transcriptional brake to restrain inflammation. Cell 165(7):1672–1685. https://doi.org/10.1016/j.cell.2016.05.075

40. Stazic D, Lindell D, Steglich C (2011) Antisense RNA protects mRNA from RNase E degradation by RNA-RNA duplex formation during phage infection. Nucleic Acids Res 39(11):4890–4899. https://doi.org/10.1093/nar/gkr037

41. Huang B, Song JH, Cheng Y, Abraham JM, Ibrahim S, Sun Z, Ke X, Meltzer SJ (2016) Long non-coding antisense RNA KRT7-AS is activated in gastric cancers and supports cancer cell progression by increasing KRT7 expression. Oncogene 35(37):4927–4936. https://doi.org/10.1038/onc.2016.25

42. Yuan S, Liu Q, Hu Z, Zhou Z, Wang G, Li C, Xie W, Meng G, Xiang Y, Wu N, Wu L, Yu Z, Bai L, Li Y (2018) Long non-coding RNA MUC5B-AS1 promotes metastasis through mutually regulating MUC5B expression in lung adenocarcinoma. Cell Death Dis 9(5):450. https://doi.org/10.1038/s41419-018-0472-6

43. Jadaliha M, Gholamalmandari O, Tang W, Zhang Y, Petrakovici A, Hao Q, Tariq A, Kim TG, Holton SE, Singh DK, Li XL, Freier SM, Amb S, Bhargava R, Lal A, Prasanth SG, Ma J, Prasanth KV (2018) A natural antisense lncRNA controls breast cancer progression by promoting tumor suppressor gene mRNA stability. PLoS Genet 14(11):e1007802. https://doi.org/10.1371/journal.pgen.1007802

44. Hao K, Lei W, Wu H, Wu J, Yang Z, Yan S, Lu XA, Li J, Xia X, Han X, Deng W, Zhong G, Zhao ZA, Hu S (2019) LncRNA-Safe contributes to cardiac fibrosis through Safe-Sfrp2-HuR complex in mouse myocardial infarction. Theranostics 9(24):7282–7297. https://doi.org/10.7150/thno.33920

45. Guo W, Liu S, Cheng Y, Lu L, Shi J, Xu G, Li N, Cheng K, Wu M, Cheng S, Liu S (2016) ICAM-1-related noncoding RNA in cancer stem cells maintains ICAM-1 expression in hepatocellular carcinoma. Clin Cancer Res 22(8):2041–2050. https://doi.org/10.1158/1078-0432.CCR-14-3106

46. Michael DR, Phillips AO, Krupa A, Martin J, Redman JE, Altabher A, Neville RD, Webber J, Kim MY, Bowen T (2011) The human hyaluronan synthase 2 (HAS2) gene and its natural antisense RNA exhibit coordinated expression in the renal proximal tubular epithelial cell. J Biol Chem 286(22):19523–19532. https://doi.org/10.1074/jbc.M111.233916

47. Faghihi MA, Zhang M, Huang J, Modarresi F, Van der Brug MP, Nalls MA, Cookson MR, St-Laurent G 3rd, Wahlestedt C (2010) Evidence for natural antisense transcript-mediated inhibition of microRNA function. Genome Biol 11(5):R56. https://doi.org/10.1186/gb-2010-11-5-r56

48. Wang GQ, Wang Y, Xiong Y, Chen XC, Ma ML, Cai R, Gao Y, Sun YM, Yang GS, Pang WJ (2016) Sirt1 AS lncRNA interacts with its mRNA to inhibit muscle formation by attenuating function of miR-34a. Sci Rep 6:21865. https://doi.org/10.1038/srep21865

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.