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Original Article

Tanshinone IIA protects motor neuron-like NSC-34 cells against lipopolysaccharide-induced cell injury by the regulation of the lncRNA TCTN2/miR-125a-5p/DUSP1 axis

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ABSTRACT

Background: Tanshinone IIA (TSIIA) exerts a protective role in spinal cord injury (SCI). However, the mechanism of TSIIA activity in SCI remains to be elucidated.
Methods: Cell viability and apoptosis were gauged by CCK-8 assay and flow cytometry, respectively. The expression levels of lncRNA TCTN2, miR-125a-5p and DUSP1 were detected by qRT-PCR and western blot. Direct relationship between miR-125a-5p and TCTN2 or DUSP1 was verified by dual-luciferase reporter assay.
Results: In mouse NSC-34 cells, LPS reduced the expression of TCTN2. TSIIA alleviated cell injury induced by LPS and increased TCTN2 expression in LPS-exposed NSC-34 cells. TCTN2 was a downstream mediator of TSIIA activity. TCTN2 targeted miR-125a-5p, and TCTN2 over-expression attenuated LPS-induced cell damage in NSC-34 cells by down-regulating miR-125a-5p. TCTN2 functioned as a post-transcriptional regulator of DUSP1 expression through miR-125a-5p. DUSP1 was a functional target of miR-125a-5p in controlling NSC-34 cell injury induced by LPS. TSIIA inhibited miR-125a-5p expression and increased the level of DUSP1 protein in LPS-exposed NSC-34 cells.
Conclusion: Our study establishes a novel mechanism, the TCTN2/miR-125a-5p/DUSP1 axis, at least in part, for the protective activity of TSIIA in cell injury induced by LPS.

1. Introduction

Spinal cord injury (SCI), a devastating condition, shows a global incidence of 10.4–83 cases per million annually and remains a common cause of death and disability, leading to a heavy economic burden to the society [1,2]. Current treatments against SCI act to reduce secondary damage [3]. Despite decades of research, effective therapeutic approaches are still limited. Therefore, developing novel therapies for the treatment of SCI is imperative.

Tanshinone IIA (TSIIA), a natural diterpene quinone extracted from the traditional Chinese herb *Salvia miltiorrhiza*, has been revealed to possess important pharmacological activities, such as anti-inflammatory, anti-oxidant, anti-angiogenic, anti-cancer, neuroprotective, and cardioprotective effects [4–6]. Several previous studies have also highlighted that TSIIA exerts a protective role in SCI [7–9]. However, the molecular determinants underlying the protective activity of TSIIA in SCI largely remain to be elucidated.

As a type of non-coding RNAs (ncRNAs), long ncRNAs (lncRNAs) are the most prevalent and functionally diverse class [10]. Work from the past decade has identified lncRNAs as critical regulators in human diseases by inhibiting microRNA (miRNA) activity [11]. Aberrant expression of lncRNAs has been demonstrated to be implicated in the pathology of SCI [12,13]. For instance, the inhibition of brain-derived neurotrophic factor antisense (BDNF-AS), an over-expressed lncRNA in SCI, impedes hypoxia-evoked neuron apoptosis by increasing miR-130b-5p abundance [14]. The deficiency of nuclear-enriched abundant transcript 1 (NEAT1) can

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improve the hind limb motor function and diminish neuronal apoptosis of SCI rats [15]. As for lncRNA tectonic family member 2 (TCTN2), encoded by the TCTN2 gene which is associated with neuronal apoptosis [16], it has established a protective role in SCI through the regulation of the miR-216b/Beclin-1 axis [17].

Recent reports have unveiled that TSIIA exerts a protective function in human diseases by controlling the expression of several lncRNAs, such as growth arrest specific 5 (GAS5) and AK003290 [18,19]. Furthermore, it remains unexplored whether lncRNA TCTN2 is responsible for TSIIA activity in protecting against SCI.

Here, we confirmed that TSIIA protected motor neuron-like NSC-34 cells against lipopolysaccharide (LPS)-induced cytotoxicity. Further, we identified the molecular basis underlying the protective effect of TSIIA on SCI.

2. Materials and methods

2.1. Cell culture and treatment

The mouse motor neuron-like NSC-34 cell line was provided by Cedarlane (Burlington, NC, USA) and cultivated at 37 °C, 5% CO_2 in Dulbecco's modified Eagle Medium (Life Technologies, Scotland, UK) plus 1% penicillin/streptomycin (Life Technologies) and 10% fetal calf serum (Biosera, Boussens, France). For LPS experiments, NSC-34 cells were maintained in a complete growth medium containing the indicated concentrations (0, 25, 50, 75 and 100 ng/mL) of LPS from *E.coli* strain Q111:B4 (Invitrogen, Hemel Hempstead, UK) for 24 h. For TSIIA experiments, NSC-34 cells were cultured for 24 h in a medium containing various doses (0, 2, 4, 6 and 8 μM) of TSIIA (98% purity, dissolved in 0.1% dimethyl sulfoxide) from AdooQ Bioscience (Irvine, CA, USA).

2.2. Transient transfection of cells

TCTN2-siRNA (si-TCTN2, 5′-AUGUUCUUCCACAUUCACGUU-3′) and dual-specificity protein phosphatase-1 (DUSP1)-siRNA (si-DUSP1, 5′-AUAUUGGUCCCGAAUGUGCCG-3′) were designed by HanBio (Shanghai, China) to silence TCTN2 and DUSP1, respectively. The non-target siRNA (si-NC, 5′-AAGACAUUGUGUGUCCGCCTT-3′, HanBio) served as a negative control. Chemically modified sequence of mouse mature miR-125a-5p (miR-125a-5p mimic, 5′-AGUGUCCAAUUUCCCAGAGUCCCU-3′, HanBio) was used to mimic miRNA expression. Modified antisense nucleotide against miR-125a-5p (miR-125a-5p inhibitor, 5′-AGGGACUCUGGGAAAUUGGACACU-3′, HanBio) was designed to knock down miR-125a-5p. Chemically modified scrambled oligonucleotides (miRNA NC mimic, 5′-ACGUGACACGUUCGGAGAATT-3′ and inhibitor NC, 5′-CAGUACUUUUGUGUAGUACAA-3′) were obtained from HanBio and used as negative controls. Mouse TCTN2 sequence incorporated with EcoR V and Xho I restriction enzyme sites was obtained from GeneCreate (Wuhan, China) and inserted into the pcDNA3.1 vector (Invitrogen) digested with EcoR V and Xho I to generate TCTN2 overexpression plasmid (pc-TCTN2), and the non-target pcDNA plasmid (pc-NC) served as the control. DUSP1 expressing plasmid (pc-DUSP1) was purchased from VectorBuilder (Guangzhou, China).

NSC-34 cells (1×10^5) were transiently transfected using Lipofectamine 3000 (Invitrogen) with 100 nM of siRNA or/and 50 nM of miRNA mimic or inhibitor or/and 200 ng of plasmid as per the manufacturing protocols. 24 h later, cells were plated for further experiments or harvested to gauge TCTN2, miR-125a-5p or DUSP1 expression.

2.2.1. Cell viability assay

Transfected or un-transfected NSC-34 cells (1×10^4 cells/well) were plated into 96-well plates and subsequently exposed to the indicated concentrations of LPS or/and TSIIA for 24 h. The number of viable cells was assayed using the Cell Counting Kit-8 (CCK-8, Biotech, Nanjing, China) by reading absorbance at a wavelength of 450 nm with a Viktor X3 reader (Perkin Elmer, Turku, Finland). Cell viability was expressed as a percentage of control cells.

2.3. Flow cytometry for cell apoptosis

Transfected or un-transfected NSC-34 cells were plated at 5×10^5 cells per well in 24-well plates and then conducted LPS or/and TSIIA treatment for 24 h. After that, cells were washed using phosphate buffered saline (PBS, Solarbio, Beijing, China), stained with Annexin V labeled by fluorescein isothiocyanate (FITC) (BD Biosciences, Heidelberg, Germany) and propidium iodide (PI, Life Technologies) for 15 min, and analyzed within 1 h. These experiments were done in triplicate and 10,000 gated events per sample were counted using the BD Biosciences flow cytometer and AccuriC6 software (BD Biosciences).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β production levels in cultured cells were evaluated by ELISA with the TNF-α Mouse ELISA Kit (Invitrogen), IL-6 Mouse ELISA Kit (Invitrogen), and IL-1β Mouse ELISA Kit (Abcam, Cambridge, UK), respectively, as per the accompanying guidance.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was prepared using the RNeasy Mini Kit as recommended by the manufacturers (Qiagen, Crawley, UK). To measure TCTN2 and DUSP1 expression, single-stranded cDNA was made in a 25 μL reaction using the ReverTra Ace RT Kit (Toyobo, Tokyo, Japan) from 2 μg of RNA; qRT-PCR was conducted using SYBR® qPCR Mix (Toyobo) and designed primers for mouse (TCTN2-forward: 5′-GCCCCAACTTCTGTACCCTC-3′, TCTN2-reverse: 5′-GTAGATGGCAGCTGAGTCCC-3′, DUSP1-forward: 5′-AGTGCCTATCACGCTTCTCG-3′, DUSP1-reverse: 5′-CCTCCACAGGGATGCTCTTG -3′); and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-CCCTTAAGAGGGATGCTGCC-3′, reverse: 5′-ACTGTGCCGTTGAATTTGCC-3′) was used as a reference gene. To quantify miR-125a-5p, 1 μg of RNA was converted to cDNA by the TaqMan miRNA RT Kit (Applied Biosystems, Toronto, Canada); qRT-PCR was done using the TaqMan miRNA Assay Kit (Applied Biosystems) with primers for mouse miR-125a-5p (forward: 5′-GCCGAGTTCCCAGAGTCCCT-3′, reverse: 5′-CTCAACTGGTGTCGTGGA-3′); and the mouse U6 severed as a housekeeping gene for normalization with specific primers (forward: 5′-CGAATCCGAACCTTTCCCCA-3′, reverse: 5′-TTTGGAAAGAGGCCATGCGG -3′). All reactions were run on a qRT-PCR System (Applied Biosystems) in triplicate and analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

2.6. Measurement of superoxide dismutase (SOD) activity and malonaldehyde (MDA) level

SOD activity of cultured NSC-34 cells was gauged using the Total Superoxide Dismutase Activity Colorimetric Assay Kit (Yeasen, Shanghai, China) as per the accompanying instructions. MDA level in cultured NSC-34 cells was measured with the MDA content Assay Kit based on the protocols of manufacturers (Solarbio).

2.7. Bioinformatics and dual-luciferase reporter assay

The miRNAs that bind to TCTN2 were searched using the database starbase (http://starbase.sysu.edu.cn/). The molecular targets of miR-

125a-5p were predicted by the online software TargetScan (http://www.targetscan.org/vert_71/?tdsourcetag = s_pcqq_aiomsg). The fragments of TCTN2 and DUSP1 3′UTR encompassing the miR-125a-5p complementary sequence were synthesized by GeneCreate and individually cloned into the pMIR-REPORT vector (Ambion, Thermo Fisher Scientific, Runcorn, UK) digested with Sac I and Hind III. Mutations in the target sequence were produced by TaKaRa MutanBest (TaKaRa, Beijing, China) to alter all seven putative complementary sites. 200 ng of individual reporter plasmid and 50 ng of pRL-TK Renilla Luciferase vector (Promega, Mannheim, Germany) were transfected into NSC-34 cells (1×10^5) together with miR-125a-5p mimic or negative mimic control at 50 nM 36 h later, cells were lysed in RIPA lysis buffer (Solarbio), and 20 μL of cell lysates were used to evaluate the luciferase activity with Dual-luciferase Reporter Assay System (Promega).

2.8. Western blot

Cultured NSC-34 cells were washed with cold PBS and homogenized in RIPA lysis buffer supplemented with Complete Mini protease inhibitors (Roche, Basel, Switzerland). The Bradford reagent (Bio-Rad, Munich, Germany) was used to examine protein concentration. Equivalent amounts of protein (50 μg per lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto Clear Blot membrane-p (ATTO, Tokyo, Japan). The primary antibodies, anti-rabbit or anti-mouse immunoglobulin secondary antibody coupled by horseradish peroxidase (ab6721 and ab6789, Abcam; dilution 1:5000), and enhanced chemiluminescence (Life Technologies) were used before densitometry analysis using the Carestream v5.2 program (Carestream Health, Rochester, NY, USA). Rabbit monoclonal DUSP1 antibody (MA5-32480; dilution 1:1000) was obtained from Invitrogen. Mouse monoclonal proliferating cell nuclear antigen (PCNA) antibody (ab29; dilution 1:1000), rabbit polyclonal B cell lymphoma-2 (Bcl-2) antibody (ab194583; dilution 1:2000), rabbit monoclonal Bax antibody (ab32503; dilution 1:3000), and rabbit monoclonal GAPDH antibody (ab181602; dilution 1:10,000) were purchased from Abcam.

2.9. Statistical analysis

All experiments were conducted at least 3 independent biological replicates, and data were plotted as mean ± standard deviation. Differences were compared using the analysis of variance (ANOVA) followed by Tukey's post hoc analysis. The significance was accepted at P less than 0.05.

3. Results

3.1. LPS inhibits the expression of TCTN2 in NSC-34 cells

We firstly evaluated the impact of LPS on cell viability, apoptosis, and inflammatory cytokines production. By contrast, LPS suppressed cell viability (Fig. 1A) and promoted cell apoptosis (Fig. 1B) in NSC-34 cells. Moreover, LPS led to increased levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in NSC-34 cells (Fig. 1C). Interestingly, LPS resulted in a remarkable down-regulation of TCTN2 expression in NSC-34 cells (Fig. 1D). All these

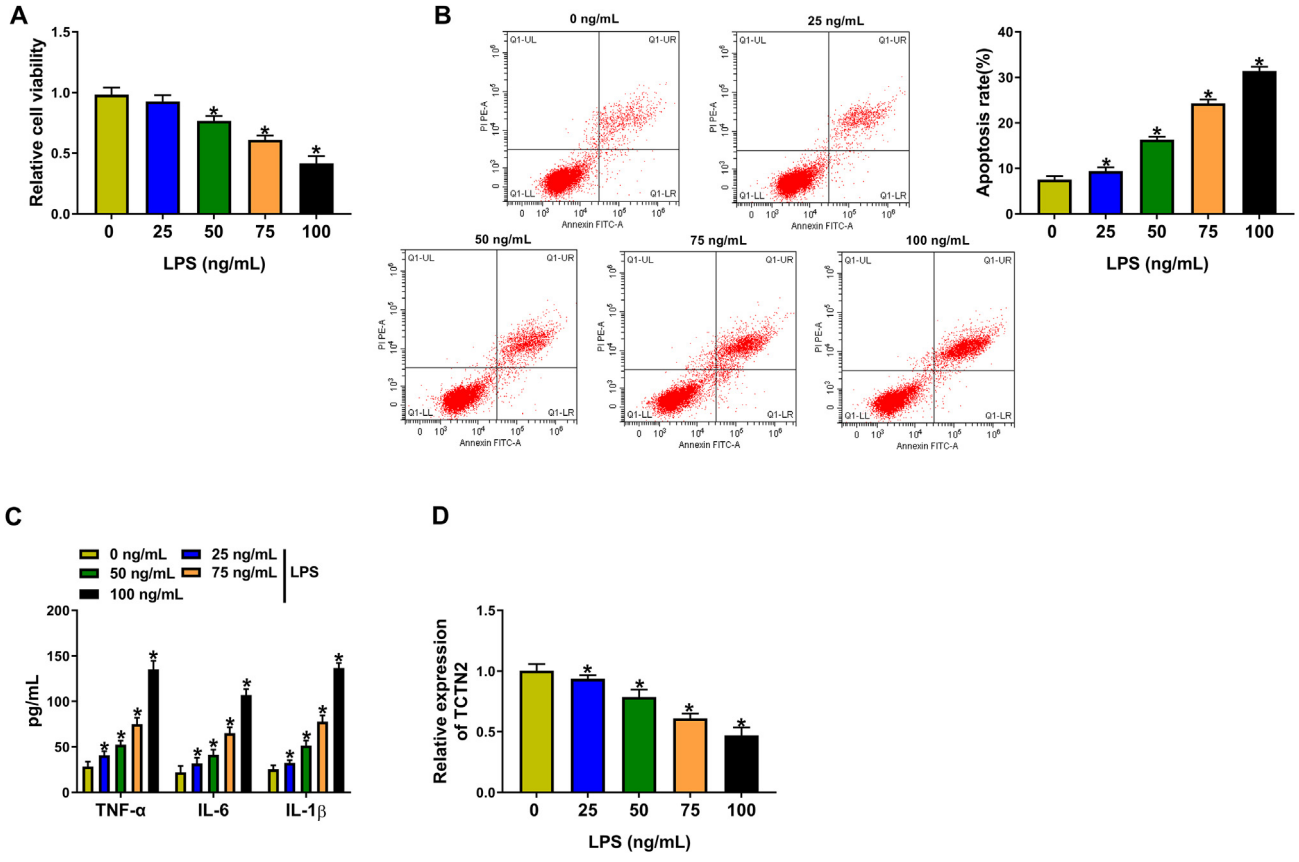

Fig. 1. LPS induces cell injury and inhibits TCTN2 expression in NSC-34 cells. NSC-34 cells were treated with LPS for 24 h at various concentrations of 0, 25, 50, 75 and 100 ng/mL. (A) Cell viability by CCK-8 assay. (B) Representative images depicting a cell apoptosis assay and cell apoptosis by flow cytometry. (C) The levels of TNF-α, IL-6 and IL-1β by ELISA assay using the assay kits. (D) qRT-PCR for relative TCTN2 expression in treated cells. LPS: lipopolysaccharide. *$P < 0.05$.

data indicated that LPS induced cell injury and reduced TCTN2 expression in NSC-34 cells.

3.2. TSIIA attenuates cell injury induced by LPS in NSC-34 cells

To elucidate the effect of TSIIA on LPS-induced cell damage, we treated NSC-34 cells with various doses of TSIIA under LPS exposure. Remarkably, TSIIA treatment enhanced cell viability (Fig. 2A) and impeded cell apoptosis (Fig. 2B) in LPS-exposed NSC-34 cells. Furthermore, TSIIA treatment resulted in decreased levels of TNF-α, IL-6 and IL-1β in NSC-34 cells under LPS exposure (Fig. 2C). To evaluate whether TCTN2 was correlated with TSIIA activity, we used qRT-PCR to gauge the expression of TCTN2 in TSIIA-treated NSC-34 cells under LPS exposure. Intriguingly, by contrast, TSIIA treatment induced a significant up-regulation in the expression of TCTN2 in LPS-exposed NSC-34 cells (Fig. 2D). Together, these results established the notion that TSIIA protected NSC-34 cells against LPS-induced cell damage by enhancing cell viability and suppressing cell apoptosis and inflammation.

3.3. TCTN2 is responsible for TSIIA activity in LPS-induced NSC-34 cells

Having demonstrated the promotion of TCTN2 expression by TSIIA, we sought to elucidate whether the up-regulation of TCTN2 was responsible for TSIIA activity. To address this possibility, we reduced TCTN2 expression using siRNA targeting TCTN2 (si-TCTN2) in NSC-34 cells (Fig. 3A). As expected, the down-regulation of TCTN2 dramatically abolished TSIIA-mediated viability promotion (Fig. 3B) and apoptosis repression (Fig. 3C) in LPS-exposed NSC-34 cells. Furthermore, the reduced expression of TCTN2 significantly reversed TSIIA-driven inhibition of TNF-α, IL-6 and IL-1β production of LPS-exposed NSC-34 cells (Fig. 3D). Additionally, the down-regulation of TCTN2 strongly counteracted the impact of TSIIA on PCNA expression, Bcl-2 level (Fig. 3E), SOD activity (Fig. 3F), as well as MDA level (Fig. 3G) in LPS-exposed NSC-34 cells. These results together suggested that TCTN2 was crucial for TSIIA function in LPS-induced NSC-34 cells.

3.4. TCTN2 targets miR-125a-5p by directly binding to miR-125a-5p

To further understand the role of TCTN2, we used the online database starbase to predict its targeted miRNAs based on the presence of the complementary sites. Bioinformatic analysis revealed a putative binding sequence for miR-125a-5p in TCTN2 (Fig. 4A). To determine the direct relationship between miR-125a-5p and TCTN2, we generated a TCTN2 luciferase reporter construct (WT-TCTN2) and transfected it into NSC-34 cells together with miR-125a-5p mimic. The transfection efficiency of miR-125a-5p mimic was gauged by qRT-PCR (Fig. 4B). With WT-TCTN2 and miR-125a-5p over-expression caused a striking down-regulation in luciferase activity (Fig. 4C). To validate whether the target sites were required for this effect, a mutant TCTN2 reporter construct (MUT-TCTN2), in which all seven putative miR-125a-5p interaction sites were mutated, was tested. Notably, this mutant no longer elicited such effect (Fig. 4C). Moreover, LPS induced the expression of miR-125a-5p in NSC-34 cells (Fig. 4D). NSC-34 cells treated by TSIIA and LPS showed lower levels of miR-125a-5p compared with the LPS control group (Fig. 4E). The transfection efficiency of TCTN2 over-expression

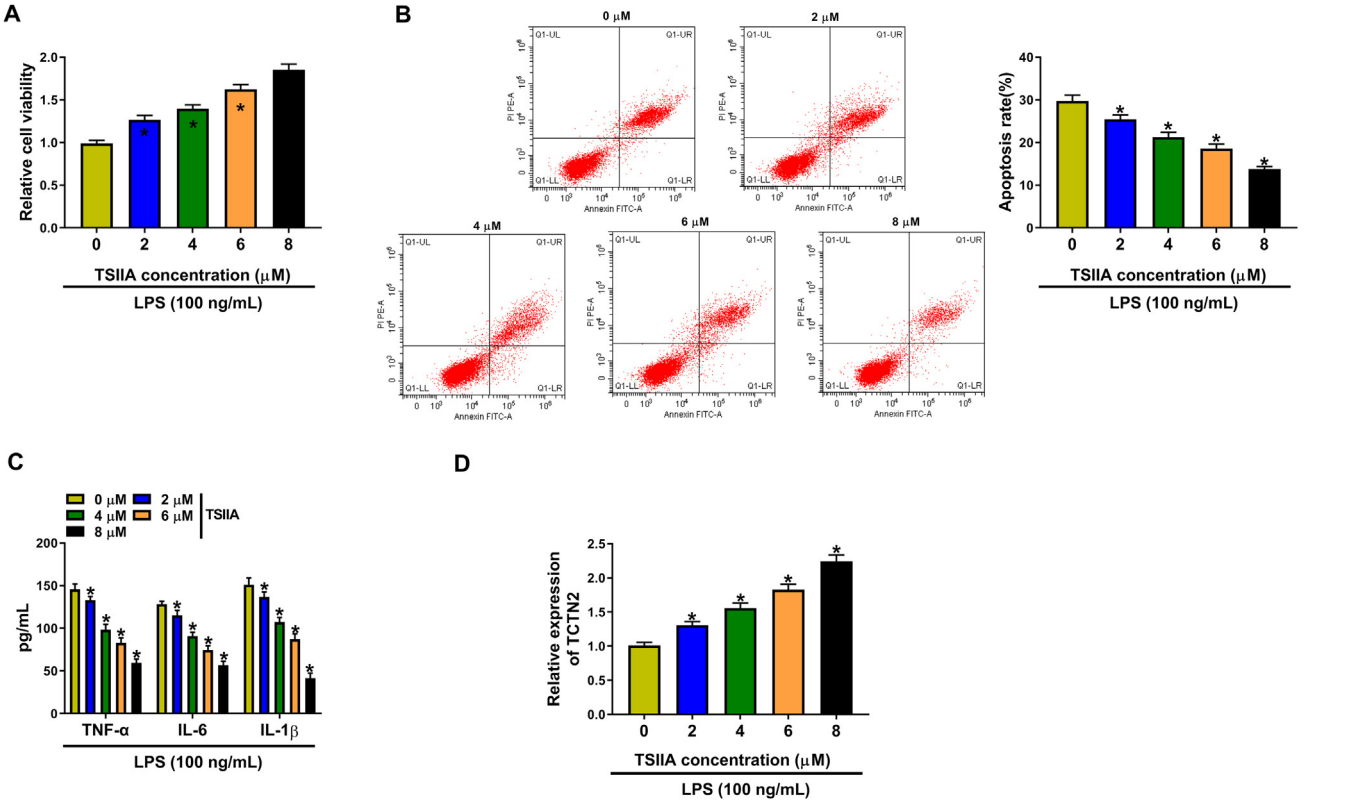

Fig. 2. TSIIA alleviates cell damage induced by LPS in NSC-34 cells. NSC-34 cells were simultaneously treated with 100 ng/mL of LPS and the indicated doses (0, 2, 4, 6 and 8 μM) of TSIIA for 24 h. (A) CCK-8 assay for the viability of cells after various treatments. (B) Representative images showing a cell apoptosis assay and cell apoptosis by flow cytometry. (C) ELISA assay for TNF-α, IL-6 and IL-1β levels using the corresponding assay kit. (D) Relative expression of TCTN2 by qRT-PCR in treated NSC-34 cells. LPS: lipopolysaccharide, TSIIA: Tanshinone IIA.*$P < 0.05$.

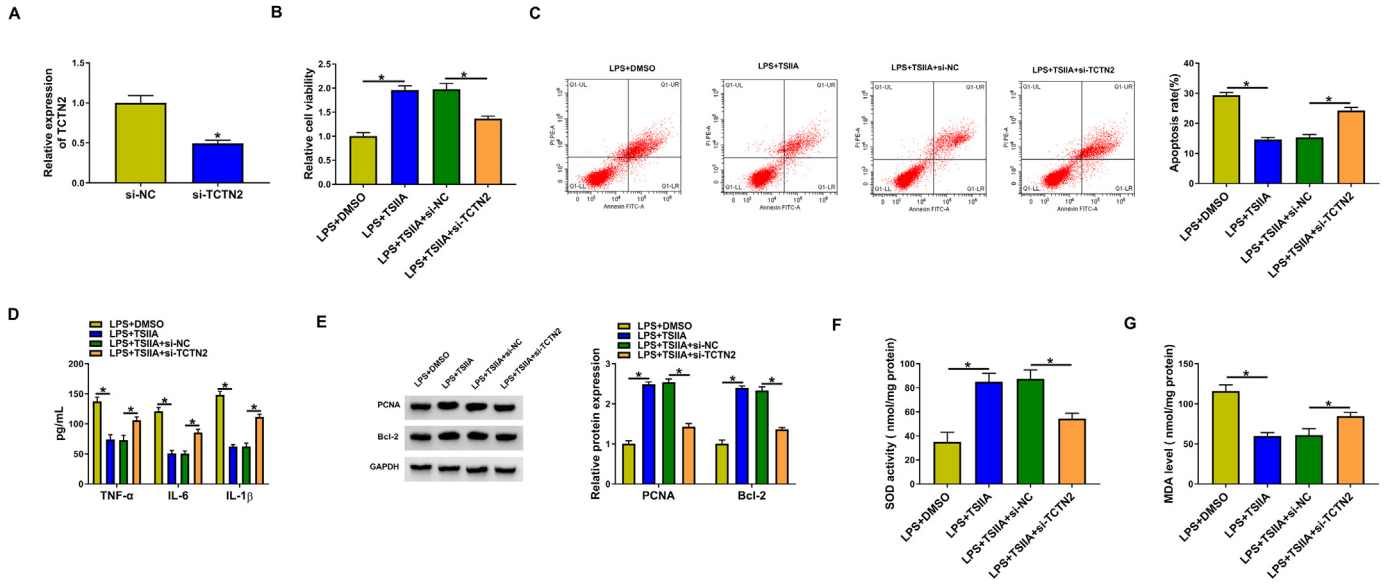

Fig. 3. TSIIA alleviates LPS-induced cell injury by elevating TCTN2 expression. NSC-34 cells were transfected with or without si-NC or si-TCTN2 and then treated with or without LPS (100 ng/mL) and TSIIA (0 or 8 μM). (A) TCTN2 expression by qRT-PCR in transfected cells. (B) Cell viability by CCK-8 assay. (C) Representative images showing a cell apoptosis assay and cell apoptosis by flow cytometry. (D) ELISA assay for TNF-α, IL-6 and IL-1β levels using the corresponding assay kit. (E) Representative images depicting a western blot assay and the levels of PCNA and Bcl-2 by western blot. (F) SOD activity using the assay kit. (G) MDA level using the assay kit. LPS: lipopolysaccharide, TSIIA: Tanshinone IIA. $^*P < 0.05$.

plasmid (pc-TCTN2) was evaluated by qRT-PCR (Fig. 4F). More interestingly, in LPS-exposed NSC-34 cells, the enforced expression of TCTN2 led to a significant inhibition in the level of miR-125a-5p, and this effect was reversed by the transfection of miR-125a-5p mimic (Fig. 4F). These results together suggested that TCTN2 directly targeted miR-125a-5p in NSC-34 cells.

3.5. Enforced expression of TCTN2 alleviates LPS-induced cell injury in NSC-34 cells by down-regulating miR-125a-5p.

We then undertook to determine how TCTN2 influenced cell injury caused by LPS in NSC-34 cells. To address this, we established TCTN2 overexpressing cells by introducing TCTN2 over-expression plasmid (pc-TCTN2) before LPS stimulation. By contrast, the enforced expression of TCTN2 enhanced cell viability (Fig. 5A) and hindered cell apoptosis (Fig. 5B) in LPS-exposed NSC-34 cells. Moreover, the over-expression of TCTN2 remarkably decreased the levels of TNF-α, IL-6 and IL-1β (Fig. 5C), as well as elevated PCNA and Bcl-2 expression (Fig. 5D) in LPS-exposed NSC-34 cells. Additionally, the enforced expression of TCTN2 led to a clear increase in the activity of SOD (Fig. 5E) and a distinct reduction in the level of MDA (Fig. 5F) in LPS-exposed NSC-34 cells. All these results strongly suggested that the enforced expression of TCTN2 protected NSC-34 cells against LPS-induced cytotoxicity.

To direct demonstrate whether TCTN2 function in LPS-exposed NSC-34 cells was mediated by miR-125a-5p, we transfected miR-125a-5p mimic in TCTN2-overexpressing cells before LPS exposure. Strikingly, the restored expression of miR-125a-5p counteracted TCTN2 over-expression-mediated cell viability enhancement (Fig. 5A) and apoptosis inhibition (Fig. 5B) in LPS-exposed NSC-34 cells. Furthermore, miR-125a-5p expression restoration markedly abolished the impact of TCTN2 over-expression on TNF-α, IL-6 and IL-1β production (Fig. 5C), PCNA and Bcl-2 expression levels (Fig. 5D), SOD activity (Fig. 5E), as well as MDA level (Fig. 5F) in LPS-exposed NSC-34 cells. Taken together, these data strongly established the notion that TCTN2 over-expression alleviated LPS-evoked cell damage through miR-125a-5p.

3.6. TCTN2 modulates DUSP1 expression through miR-125a-5p

To identify the molecular targets of miR-125a-5p, we used the mRNA-target-prediction software TargetScan. Intriguingly, a putative complementary sequence for miR-125a-5p was found in the 3′UTR of DUSP1 (Fig. 6A). To demonstrate this, we generated DUSP1 3′UTR luciferase reporter (WT-DUSP1 3′UTR) and site-directed mutation in the binding sequence (MUT-DUSP1 3′UTR) and tested them by dual-luciferase reporter assays. Notably, the luciferase activity of WT-DUSP1 3′UTR was repressed by miR-125a-5p over-expression, while MUT-DUSP1 3′UTR was refractory to the suppression by miR-125a-5p (Fig. 6B). In addition, LPS exposure in NSC-34 cells resulted in a strong reduction in the protein level of DUSP1 (Fig. 6C). Moreover, TSIIA treatment strikingly increased the protein expression of DUSP1 in NSC-34 cells under LPS exposure (Fig. 6D). The transfection efficiencies of si-DUSP1 and miR-125a-5p inhibitor were validated by western blot and qRT-PCR, respectively (Fig. 6E and F). By contrast, in LPS-exposed NSC-34 cells, the down-regulation of miR-125a-5p resulted in increased protein level of DUSP1, and this effect was reversed by the transfection of si-DUSP1 (Fig. 6G). We next determined whether TCTN2 modulated DUSP1 expression in LPS-exposed NSC-34 cells. As would be expected, the enforced expression of TCTN2 led to a significant increase in the protein level of DUSP1, and this effect was remarkably abrogated by miR-125a-5p up-regulation in NSC-34 cells under LPS exposure (Fig. 6H). Together, these results pointed to the role of TCTN2 as a regulator of DUSP1 expression through miR-125a-5p.

3.7. DUSP1 is a functional target of miR-125a-5p in regulating NSC-34 cell injury induced by LPS

We next elucidated the mechanistic insight into the link between miR-125a-5p and DUSP1 in regulating cell injury induced by LPS. Remarkably, the reduced expression of miR-125a-5p promoted cell viability (Fig. 7A), and repressed cell apoptosis (Fig. 7B), as well as inhibited the production of TNF-α, IL-6 and IL-1β (Fig. 7C) in LPS-exposed NSC-34 cells. Moreover, the down-regulation of miR-125a-

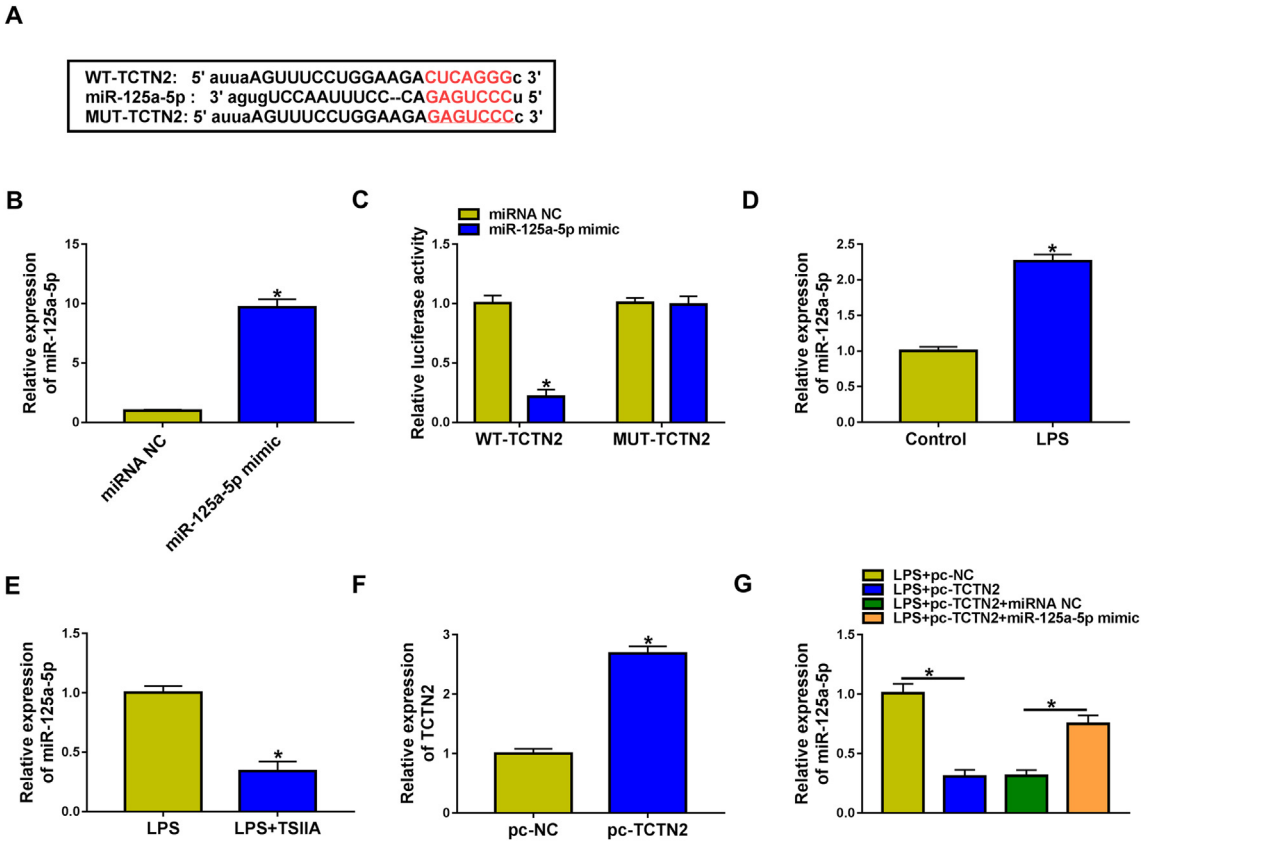

Fig. 4. TCTN2 directly targets miR-125a-5p. (A) Sequence of miR-125a-5p, the binding sites for miR-125a-5p in TCTN2 and the mutation of the target sites. (B) qRT-PCR for miR-125a-5p relative expression in NSC-34 cells transfected with miRNA NC mimic or miR-125a-5p mimic. (C) Dual-luciferase reporter assays in NSC-34 cells with TCTN2 luciferase reporter construct (WT-TCTN2) or mutant TCTN2 reporter construct (MUT-TCTN2). (D) Relative expression of miR-125a-5p by qRT-PCR in NSC-34 cells exposed to 100 ng of LPS or control for 24 h. (E) qRT-PCR for miR-125a-5p expression in NSC-34 cells treated with LPS or LPS + TSIIA (8 μM). (F) TCTN2 expression by qRT-PCR in NSC-34 cells transfected with pc-NC or pc-TCTN2 (TCTN2 overexpression plasmid). (G) The level of miR-125a-5p by qRT-PCR in NSC-34 cells transfected with pc-NC, pc-TCTN2 (TCTN2 overexpression plasmid), pc-TCTN2+miRNA NC mimic or pc-TCTN2+miR-125a-5p mimic before LPS exposure. LPS: lipopolysaccharide, TSIIA: Tanshinone IIA. *$P < 0.05$.

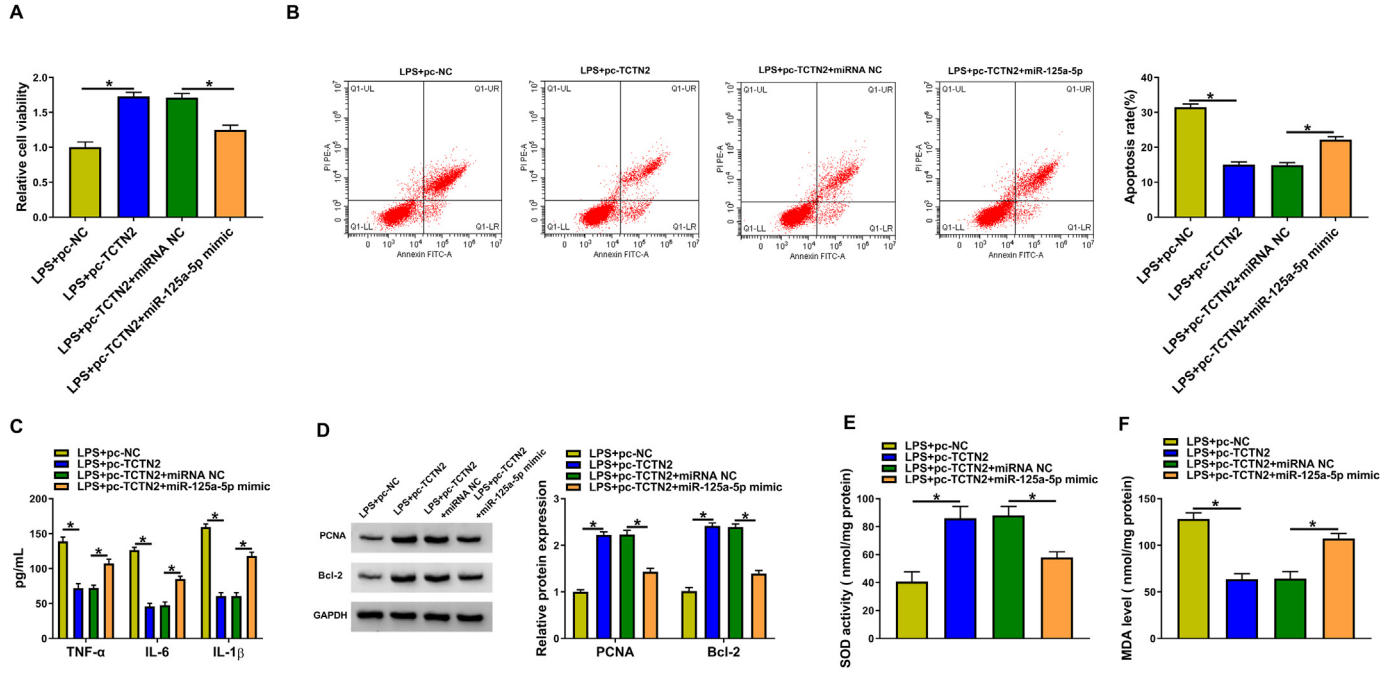

Fig. 5. Enforced expression of TCTN2 alleviates LPS-induced cell injury through miR-125a-5p. NSC-34 cells were transfected with pc-NC, pc-TCTN2 (TCTN2 overexpression plasmid), pc-TCTN2+miRNA NC mimic or pc-TCTN2+miR-125a-5p mimic and then exposed to 100 ng/mL of LPS for 24 h. (A) CCK-8 assay for cell viability. (B) Representative images showing a cell apoptosis assay and cell apoptosis by flow cytometry. (C) ELISA assay for TNF-α, IL-6 and IL-1β levels using the corresponding assay kit. (D) Representative images depicting a western blot assay and the levels of PCNA and Bcl-2 by western blot. (E) SOD activity using the assay kit. (F) MDA level using the assay kit. LPS: lipopolysaccharide. *$P < 0.05$.

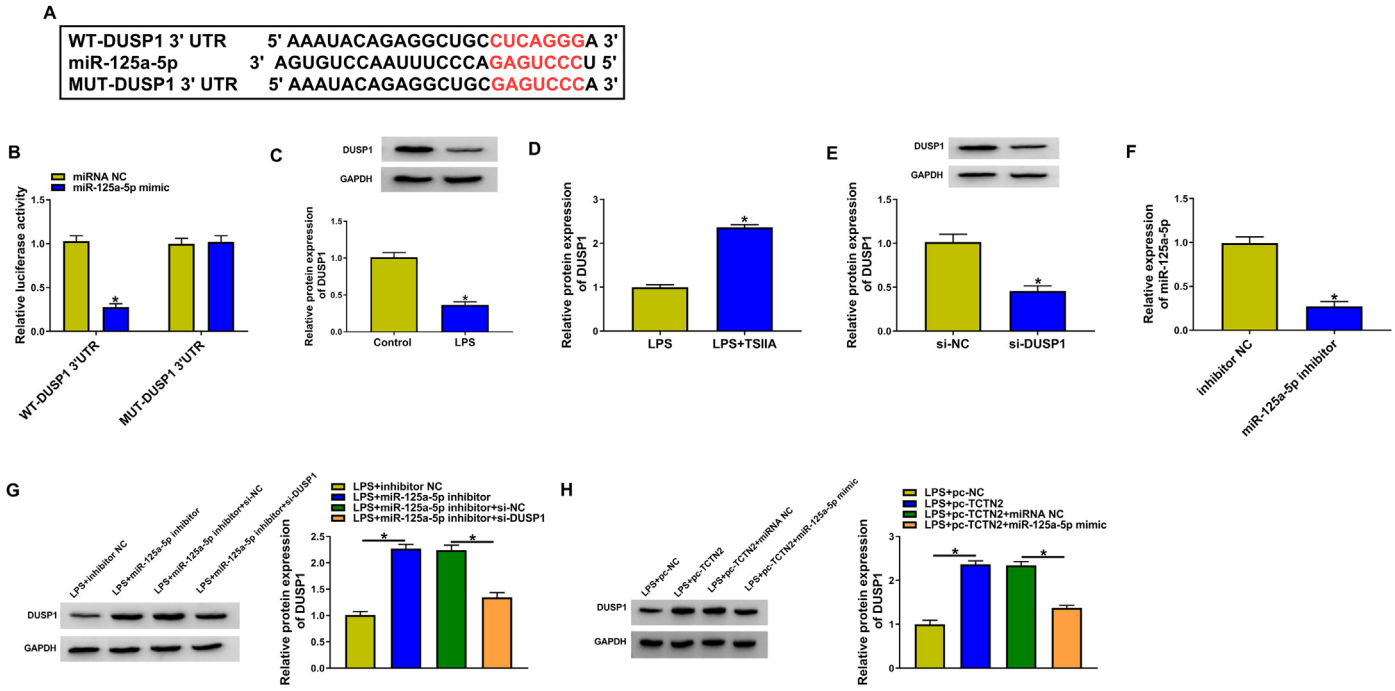

Fig. 6. TCTN2 targets miR-125a-5p to regulate the expression of DUSP1. (A) Sequence of miR-125a-5p, the binding sites for miR-125a-5p in DUSP1 3′UTR and the mutation of the target sites. (B) Dual-luciferase reporter assays in NSC-34 cells with DUSP1 3′UTR luciferase reporter construct (WT-DUSP1 3′UTR) or mutant DUSP1 3′UTR reporter construct (MUT-DUSP1 3′UTR). (C) Relative expression of DUSP1 protein by western blot in NSC-34 cells exposed to 100 ng/mL of LPS or control for 24 h. (D) Western blot for DUSP1 protein level in NSC-34 cells treated with LPS or LPS + TSIIA (8 μM). (E) DUSP1 protein level by western blot in NSC-34 cells transfected with si-NC or si-DUSP1. (F) qRT-PCR for miR-125a-5p expression in NSC-34 cells transfected with inhibitor NC or miR-125a-5p inhibitor. (G) Western blot for DUSP1 protein level in LPS-exposed NSC-34 cells transfected with inhibitor NC, miR-125a-5p inhibitor, miR-125a-5p inhibitor + si-NC or miR-125a-5p inhibitor + si-DUSP1. (H) DUSP1 protein level by western blot in LPS-treated NSC-34 cells transfected with pc-NC, pc-TCTN2 (TCTN2 overexpression plasmid), pc-TCTN2+miRNA NC mimic or pc-TCTN2+miR-125a-5p mimic. LPS: lipopolysaccharide, TSIIA: Tanshinone IIA. $^*P < 0.05$.

5p in LPS-exposed NSC-34 cells led to a strong elevation in the levels of PCNA and Bcl-2 (Fig. 7D), a clear enhancement in SOD activity (Fig. 7E), as well as an obvious inhibition in MDA level (Fig. 7F) compared with the control group. Furthermore, these effects of miR-125a-5p depletion were dramatically abolished by the down-regulation of DUSP1 in LPS-exposed NSC-34 cells (Fig. 7A–F). Additionally, in LPS-exposed NSC-34 cells, increased expression of DUSP1 suppressed cell apoptosis and reduced the level of pro-apoptosis protein Bax, as well as elevated Bcl-2 expression (Supplement Fig. 1). All these results suggested that the regulation of miR-125a-5p in LPS-induced NSC-34 cells was mediated by DUSP1.

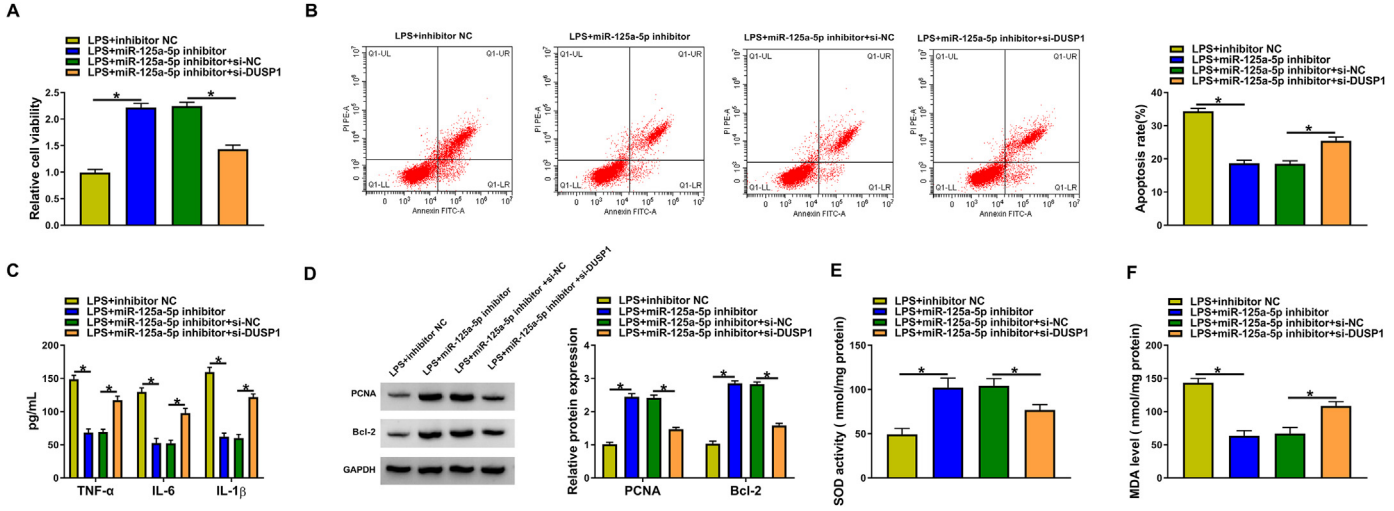

Fig. 7. The reduced expression of miR-125a-5p alleviates LPS-induced cell damage by up-regulating DUSP1. NSC-34 cells were transfected with inhibitor NC, miR-125a-5p inhibitor, miR-125a-5p inhibitor + si-NC or miR-125a-5p inhibitor + si-DUSP1 and then exposed to 100 ng/mL of LPS for 24 h. (A) Cell viability by CCK-8 assay. (B) Representative images showing a cell apoptosis assay and cell apoptosis by flow cytometry. (C) ELISA assay for TNF-α, IL-6 and IL-1β levels using the corresponding assay kit. (D) Representative images depicting a western blot assay and the levels of PCNA and Bcl-2 by western blot. (E) SOD activity using the assay kit. (F) MDA level using the assay kit. LPS: lipopolysaccharide. $^*P < 0.05$.

4. Discussion

Recently, the protective effect of TSIIA against SCI has been widely reported [7–9,20]. However, our understanding of its molecular basis is still limited. Here, we used a motor neuron-like NSC-34 cell line induced by LPS to mimic the *in vitro* SCI cell model [21]. Our results validated that TSIIA protected NSC-34 cells against LPS-induced cell damage, in line with recent work [22,23]. Importantly, we provided a novel molecular explanation for the protective role of TSIIA in LPS-evoked cytotoxicity in NSC-34 cells. Additionally, our data showed that when TSIIA treatment was at high concentrations (14 and 20 μM), cell viability was suppressed and cell apoptosis was promoted in LPS-treated NSC-34 cells (Supplement Fig. 2). Although the therapeutic potential of TSIIA has been widely reported in human diseases [24], our findings unveiled its toxic activity in LPS-treated NSC-34 cells, consistent with the previous studies [25,26], which may help avoid the risk of its clinical application.

TCTN2, a remarkably under-expressed lncRNA in the spinal cord tissues of the SCI rat model, can repress neuronal apoptosis by promoting autophagy [17]. Interestingly, we found that TSIIA upregulated TCTN2 expression in LPS-exposed NSC-34 cells, which prompted us to examine whether TCTN2 was responsible for TSIIA activity. Here we demonstrated, for the first time, that TCTN2 was crucial for TSIIA activity, which was also confirmed by the alteration of SOD activity and MDA level, two indicators of the activation of oxidative stress [27]. Similarly, several other lncRNAs, such as ANRIL and HOTAIR, have been shown to be involved in the protective activity of TSIIA in cell injury [19,28].

MiR-125a-5p has been implicated in the pathogenesis of various diseases, such as acute ischemic stroke and type 2 diabetes mellitus [29,30]. Moreover, altered expression of miR-125a-5p has been reported to impact human carcinogenesis [31–33]. Interestingly, a previous report uncovered the crucial involvement of miR-125a-5p in H_2O_2-induced cell injury during SCI [34]. Here, we first identified that TCTN2 directly targeted miR-125a-5p, and TCTN2 overexpression alleviated LPS-induced NSC-34 cell injury by downregulating miR-125a-5p. Importantly, we first ascertained that TSIIA reduced the expression of miR-125a-5p in LPS-exposed NSC-34 cells.

DUSP1 has established a protective role in cardiac ischemia/reperfusion injury and diabetic nephropathy [35,36]. Moreover, DUSP1 is closely associated with tumor chemotherapy, radiotherapy and immunotherapy [37]. In this study, DUSP1 was a strong candidate as a miR-125a-5p target, considering its protective effect on neuronal injury in the spinal cord after brachial plexus injury [21]. Our results first showed that TSIIA induced DUSP1 expression, and miR-125a-5p regulated LPS-induced cell injury by targeting DUSP1. Similarly, Liu et al. identified the modulation of miR-101–3p in neuronal injury and neuroinflammation in the spinal cord via DUSP1 [21]. Furthermore, we first pointed to the role of TCTN2 as a post-transcriptional modulator of DUSP1 expression through miR-125a-5p. Additionally, Jin and colleagues identified DUSP1 as a protective factor in cardiac ischemia/reperfusion injury by inhibiting the JNK pathway, which plays a critical role in SCI [38]. A future challenge will be to identify whether the pathway represents a functionally downstream effector of the TSIIA/TCTN2/miR-125a-5p/DUSP1 axis in SCI. Such analysis is hampered at present by the lack of *in vivo* studies with the SCI animal models. In the current study, we used LPS-evoked NSC-34 cells as the *in vitro* model of SCI, which is limited because LPS-evoked NSC-34 cells cannot really mimic the neurons under SCI.

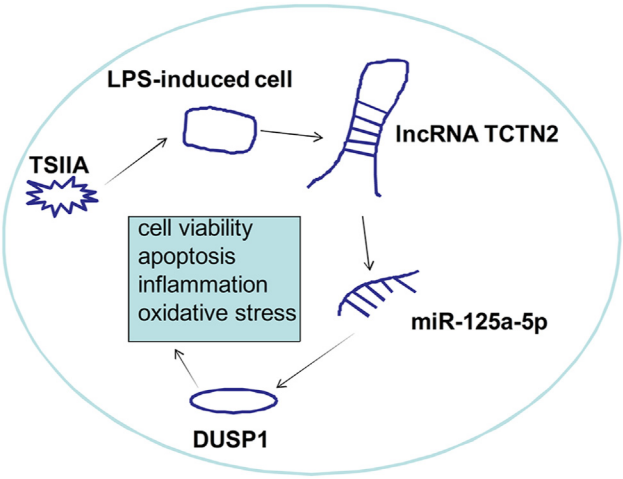

Fig. 8. Tanshinone IIA protects LPS-induced NSC-34 cell injury by regulating the lncRNA TCTN2/miR-125a-5p/DUSP1 axis.

5. Conclusions

Collectively, these findings establish the TCTN2/miR-125a-5p/DUSP1 axis as a novel molecular mechanism for the protective role of TSIIA in cell injury induced by LPS (Fig. 8). Our findings highlight a strong rationale for developing TSIIA as a promising agent against SCI.

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Disclosure of interest

The authors declare that they have no financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2023.03.007.

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