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Zhilong Huoxue Tongyu Capsule regulates the macrophage polarization and inflammatory response via the let-7i/TLR9/MyD88 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: Zhilong Huoxue Tongyu Capsule (ZL) is clinically prescribed for acute ischemic stroke (AIS). However, only a few studies have addressed the mechanisms of ZL in treating AIS.
Aim of the study: To explore the underlying mechanism of macrophage polarization and inflammation mediated by ZL, and to provide a reference for AIS treatment.
Materials and methods: Sixteen SD rats were fed with different dose of ZL (0, 0.4, 0.8, and 1.6 g/kg/d) for 4 days to prepare ZL serum. After 500 ng/mL lipopolysaccharide (LPS) stimulation, RAW264.7 cells were administrated with ZL serum. Then, experiments including ELISA, flow cytometry, real-time quantitative PCR and Western blot were performed to verify the effects of ZL on macrophage polarization and inflammation. Next, let-7i inhibitor was transfected in RAW264.7 cells when treated with LPS and ZL serum to verify the regulation of ZL on the let-7i/TLR9/MyD88 signaling pathway. Moreover, the interaction between let-7i and TLR9 was confirmed by the dual-luciferase assay.
Results: ZL serum significantly decreased the expression of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), and increased the expression of IL-10 and transforming growth factor β1 (TGF-β1) of LPS stimulated-macrophages. Furthermore, ZL serum polarized macrophages toward M2, decreased the expressions of TLR9, MyD88, and iNOS, as well as increased the expressions of let-7i, CHIL3, and Arginase-1. It is worth mentioning that the effect of ZL serum is dose-dependent. However, let-7i inhibitor restored all the above effects in LPS stimulated-macrophages. In addition, TLR9 was the target of let-7i.
Conclusions: ZL targeted let-7i to inhibit TLR9 expression, thereby inhibiting the activation of the TLR9/MyD88 pathway, promoting the M2 polarization, and inhibiting the development of inflammation in AIS.

1. Introduction

Acute ischemic stroke (AIS) is one of the most common types of stroke. As a serious complication of thrombolytic therapy in AIS, hemorrhagic transformation results in inflammatory response and M1 macrophage polarization, to further induce brain damage(Won et al., 2021). Macrophages are a crucial type of immune cell that comprises phenotypes of M1 and M2. Specifically, M1 macrophages are involved in pro-inflammation, while M2 macrophages are involved in anti-inflammation(Brifault et al., 2015). Macrophage polarization plays a key role in modulating the M1/M2 ratio under various microenvironmental stimulation and influences the occurrence and development of inflammatory responses. Evidences have demonstrated that inhibition of M1 polarization or promotion of M2 polarization is an effective way to

Abbreviations: AIS, acute ischemic stroke; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; NC, negative control; RT-qPCR, real-time quantitative PCR; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor-α; WB, western blot; ZL, Zhilong Huoxue Tongyu Capsule.

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reduce AIS-induced brain damage(Jiang et al., 2018; Yang et al., 2022).

MicroRNA is a type of small RNAs that regulates gene expression by binding mRNA to inhibit the translation of target genes(Tsuchiya et al., 2006). The let-7 family is widely distributed in biology, and numerous studies previously reported that it plays a significant regulatory role in the development of cancer(Wang et al., 2018). Let-7c inhibited the development of liver carcinoma by targeting the PI3K/Akt/FoxO pathway(Li et al., 2021) and the upregulation of let-7i suppressed the malignant phenotypes of colorectal cancer cells by inhibiting CCND1(Tu et al., 2022). Moreover, in recent years, the let-7 family has also been found to be associated with AIS, and its regulatory roles mainly involve neuroprotective and nerve regenerative functions(Gong et al., 2016; Peng et al., 2015). It is reported that let-7i was upregulated in AISpatients who received thrombolytic therapy(Xiang et al., 2017), which indicating that let-7i may be a therapeutic target for AIS, but further study is needed to support this hypothesis.

Zhilong Huoxue Tongyu Capsule (ZL) is a traditional Chinese medicine compound formulation independently developed by our hospital with the National Invention Patent No. Of 200810147774.1. ZL consists of *Astragalus membranaceus* (Fisch.) Bunge (HuangQi), *Cinnamomum cassia* (L.) J.Presl (GuiZhi), Leech (ShuiZhi), *Earthworm* (DiLong), and Sargentodoxa cuneata (Oliv.) Rehder & E.H.Wilson (DaXueTeng)(Geng et al., 2023). Previous meta-analysis that included 571 AIS patients indicated that compared with guideline-recommended medical therapy alone, ZL combined with guideline-recommended medical therapy significantly improved the clinical effective rate and decreased National Institutes of Health Stroke Scale Score, Barthel Index, and Modified Rankin Scale without reported adverse events(Liu et al., 2021). Another systematic review found that ZL effectively reduced homocysteine and inflammatory response, alleviated vascular endothelial cell dysfunction, and regulated abnormal blood lipids and blood rheology in patients with AIS(Liang et al., 2021). Mechanistically, ZL alleviates surgical neurological symptoms in the cerebral ischemia-reperfusion model and protects the brain by mediating inflammatory reactions and M2 polarization of macrophages(Bai et al., 2009; Luo et al., 2010). However, its regulatory mechanisms on macrophage polarization and inflammation in AIS are still unclear. Here, we investigated the molecular mechanisms by which ZL alleviates AIS through macrophage polarization and inflammatory response.

2. Materials and methods

2.1. ZL serum preparation

ZL was characterized by the ultra-high performance liquid chromatography coupled high resolution mass spectrometry(Mazhar et al., 2022). A total of sixteen SD rats were used for ZL serum preparation. All rats were obtained from Chengdu Dashuo Co., LTD (Chengdu, China) [SYXK(Chuan) 2015-010] and weighed 180g–220g. ZL was obtained from The Affiliated Traditional Chinese Medicine Hospital, Southwest Medical University (Luzhou, China), and four concentrations (0, 0.4, 0.8, or 1.6 g/kg/d) were fed for three consecutive days. One hour after the last administration, blood was collected from the abdominal aorta and centrifuged at 3000 r/min for 10 min to extract serums. We followed the Guidelines for the Care and Use of Animals in Research enforced by Southwest Medical University and all protocols were approved by the Institutional Review Board (No. swmu20230064).

2.2. RAW264.7 cells culture and treatment

DMEM (Procell, Wuhan, China) with 10% fetal bovine serum and 1% penicillin-streptomycin solution was used to culture RAW264.7 cells (Procell) at 37 °C and 5% CO_2. After cells grew to log-phase, cells were stimulated with 500 ng/mL lipopolysaccharide (LPS) for 12 h, and collected for the following experiments. Experiment (1): cells were treated with different concentrations of ZL serum for 12 h and divided into the Control, LPS, ZL-Con (LPS + blank serum), ZL-L (LPS + ZL-L serum), ZL-M (LPS + ZL-M serum), and ZL-H (LPS + ZL-H serum) groups; Experiment (2): cells were treated with ZL-H serum for 12 h, transfected with negative control (NC) or let-7i inhibitor through lip2000 (Invitrogen, USA), and divided into the Control, LPS, ZL (LPS + ZL-H serum), ZL + NC inhibitor (LPS + ZL-H serum + NC inhibitor), ZL + let-7i inhibitor (LPS + ZL-H serum + let-7i inhibitor) groups.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatant was collected, and cytokines including interleukin (IL)-6, IL-10, tumor necrosis factor-α (TNF-α), and transforming growth factor β1 (TGF-β1) were quantified by ELISA kits based on the manufacturers' instructions. The information of ELISA kits is listed as follows: IL-6 (ZC-32446, ZCi Bio, Shanghai China), IL-10 (ZC-37962, ZCi Bio), TNF-α (ZC-35733, ZCi Bio), and TGF-β1 (ZC-39043, ZCi Bio).

2.4. Flow cytometry assay

Six-well plates were used to seed 2×10^5 cells/well of macrophages. After drug treatment or inhibitor transfection, cells were resuspended with PBS and incubated with 0.5 μl/T F4-80 and 1.25 μl/T CD86 (Solarbio, Beijing, China) for 30 min at 4 °C. Next, after being collected and washed twice, cells were resuspended with 100 μl intracellular straining perm wash buffer and incubated with 2.5 μl/T CD206 (Solarbio) for 30 min at 4 °C. Finally, cells were analyzed by BD FACSCanto II flow cytometer (BD Biosciences, New Jersey, USA). FlowJo software (version 10.1) was used to analyze all Flow cytometry data.

2.5. Real-time quantitative PCR (RT-qPCR) assay

The total RNA of RAW264.7 cells was isolated, and the PrimeScript™ RT reagent Kit (Takara, Japan) was used to synthesize cDNA according to the manufacturer's instructions. Genes including TLR9, MyD88, let-7i, and let-7e were quantified by RT-qPCR with SYBR qPCR Master Mix (Vazyme, Nanjing, China)(Liang et al., 2022a). The primers were synthesized by BGI (Shenzhen, China) and the sequences are listed in Table 1.

2.6. Double luciferase gene report assay

A luciferase reporter assay was performed to determine the interaction between let-7i-3p and TLR9. The 3′UTR fragment of TLR9 was cloned into the pSI-Check2 vector (Hanbio, Shanghai, China), and the vector with the NC/let-7i-3p mimic were co-transfected into HEK293T cells (Procell) for 48 h. After that, Promega Dual-Luciferase system (Hanbio) was employed to detect the luciferase activity.

2.7. Western blot (WB) assay

After LPS, ZL serum, or let-7i inhibitor treatment, RAW264.7 cells were collected to separate total protein by RIPA lysis buffer (Beyotime, Shanghai, China). BCA kit (Beyotime) was used to quantify the concentration of protein and the protein was transferred to membranes as described previously(Liang et al., 2022b). After incubating with the primary antibodies containing TLR9 (1:2000, ABclonal, Wuhan, China), MyD88 (1:2000, ABclonal), iNOS (1:1000, ABclonal), Arginase-1 (1:1000, Cell Signaling Technology, Boston, USA), CHIL3 (1:1000, Thermo Fisher Scientific, Massachusetts, USA), and β-actin (1:100000, ABclonal), the membranes were washed with PBS for three times and incubated with horseradish peroxidase-conjugate anti-rabbit (1:2000, Abcam, Cambridge, UK) or anti-mouse (1:2000, Abcam) second antibodies. Finally, BeyoECL Plus (Beyotime) was used to detect the protein expression and the ImageJ software (version 1.54i) was used to express the intensity of the bands.

Table 1
Sequence of primers.

Genes	Forward (5′-3′)	Reverse (5′-3′)
Tlr9	GTTCCTGCCGCTGACCAACCTGAAG	CACCGTTGCCGCTGAAGTCCAGATAC
Myd88	TGTCGTCGCATGGTGGTGGTTGTT	AGTCGCTTCTGTTGGACACCTGGAGA
Let-7i	ACACTCCAGCTGGGCTGCGCAAGCTACTGCCT	CTCAACTGGTGTCGTGGA
Let-7e	GCCGCTTGAGGTAGGAGGTTGT	CCAGTGCAGGGTCCGAGGT
β-actin	AAAATGGCAGTGCGTTTAG	TTTGAAGGCAGTCTGTCGTA

2.8. Statistics and analysis

Graphpad Prism (version 9.0.1) was performed to analyze the data and all data in this study was represented as the mean ± standard error of mean. All experiments were repeated at least three times. Comparison between two groups was conducted using Student *t*-test, and comparison among multiple groups was conducted using one-way of variance. $P < 0.05$ was accepted as significance.

3. Results

3.1. ZL inhibited the inflammation

Inflammation plays an important role in brain damage after occurring of AIS. Here, we measured the levels of IL-6, IL-10, TNF-α, and TGF-β1 using ELISA to investigate the effects of ZL serum on RAW264.7 cell inflammation. As expected, cytokines of IL-10 (Fig. 1A) and TGF-β1 (Fig. 1B) were down-regulated by LPS, but were up-regulated by ZL with a dose-dependent manner. Conversely, cytokines of IL-6 (Fig. 1C) and TNF-α (Fig. 1D) were up-regulated by LPS, but were significantly down-regulated by ZL with a dose-dependent manner. It is suggested that ZL could inhibit inflammation of RAW264.7 cells by regulation of cytokines release. However, the molecular mechanism needs to be further studied.

3.2. ZL polarized macrophages toward M2

Macrophage polarization is an important factor determining the development of inflammation. Through flow cytometry, we labeled M1 and 2 macrophages with CD86 and CD206, respectively, and quantified them (Fig. 2A). Fig. 2B showed that LPS induced an increase of the M1/M2 ratio, while ZL decreased this ratio. In addition, the marker proteins of macrophage polarization including Arginase-1, CHIL3 and iNOS were determined by WB (Fig. 2C). The results indicated that ZL significantly reversed the LPS-induced over-expression of iNOS and down-expression of Arginase-1 and CHIL3 (Fig. 2D–F). Importantly, all effects of ZL were a dose-dependent manner.

3.3. ZL targeted let-7i to inhibit the activation of the TLR9/MyD88 pathway

Considering the role of let-7 in the AIS development, we firstly investigated the influence of ZL on the expression of let-7i and let-7e through RT-qPCR and we found that let-7i and let-7e was obviously down-regulated by LPS and up-regulated by ZL (Fig. 3A&B). It noticed that let-7i exhibited a more significant change than let-7e. Thus, let-7i was chosen for further study. Let-7 activates the RNA-sensing TLR (Lehmann et al., 2012), and we verified TLR9 was a target of let-7i through double luciferase assay (Fig. 3C&D). Next, the expressions of TLR9 and MyD88 were determined, the results revealed that ZL serum could reduce the expressions of MyD88 and TLR9 up-regulated by LPS

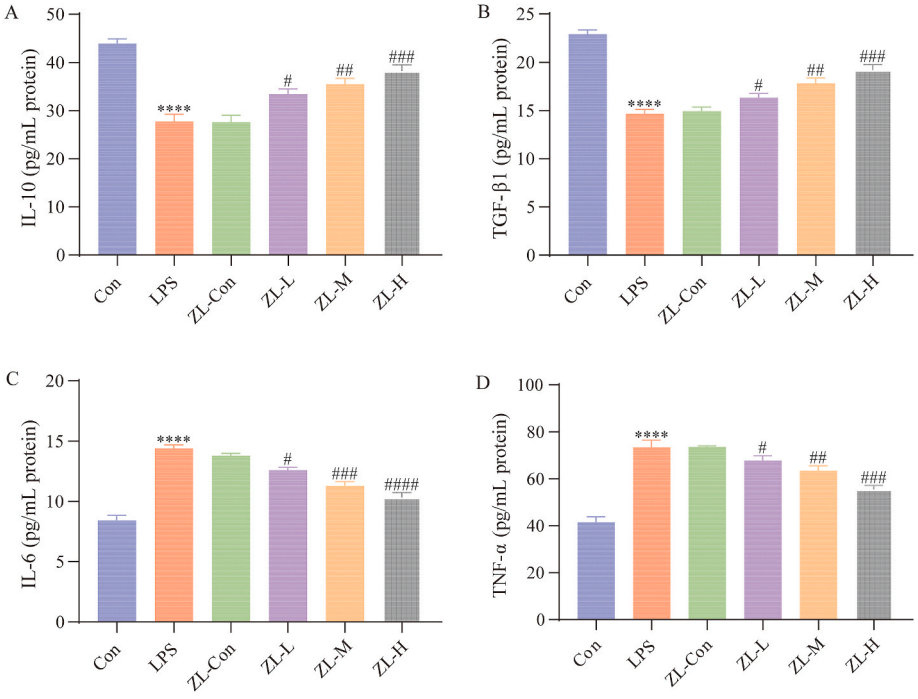

Fig. 1. ZL regulated the cytokines release of RAW264.7 cells. After LPS stimulation, RAW264.7 cells were treated with the ZL serum. ELISA was performed to measure the contents of (A) IL-10, (B) TGF-β1, (C) IL-6, and (D) TNF-α. The concentrations of ZL serum in the ZL-Con, ZL-L, ZL-M, and ZL-H groups were 0, 0.4, 0.8, and 1.6 g/kg/d, respectively. Mean ± standard error of mean was represented by bars. $^{****}P < 0.0001$ compared with the Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with the LPS group.

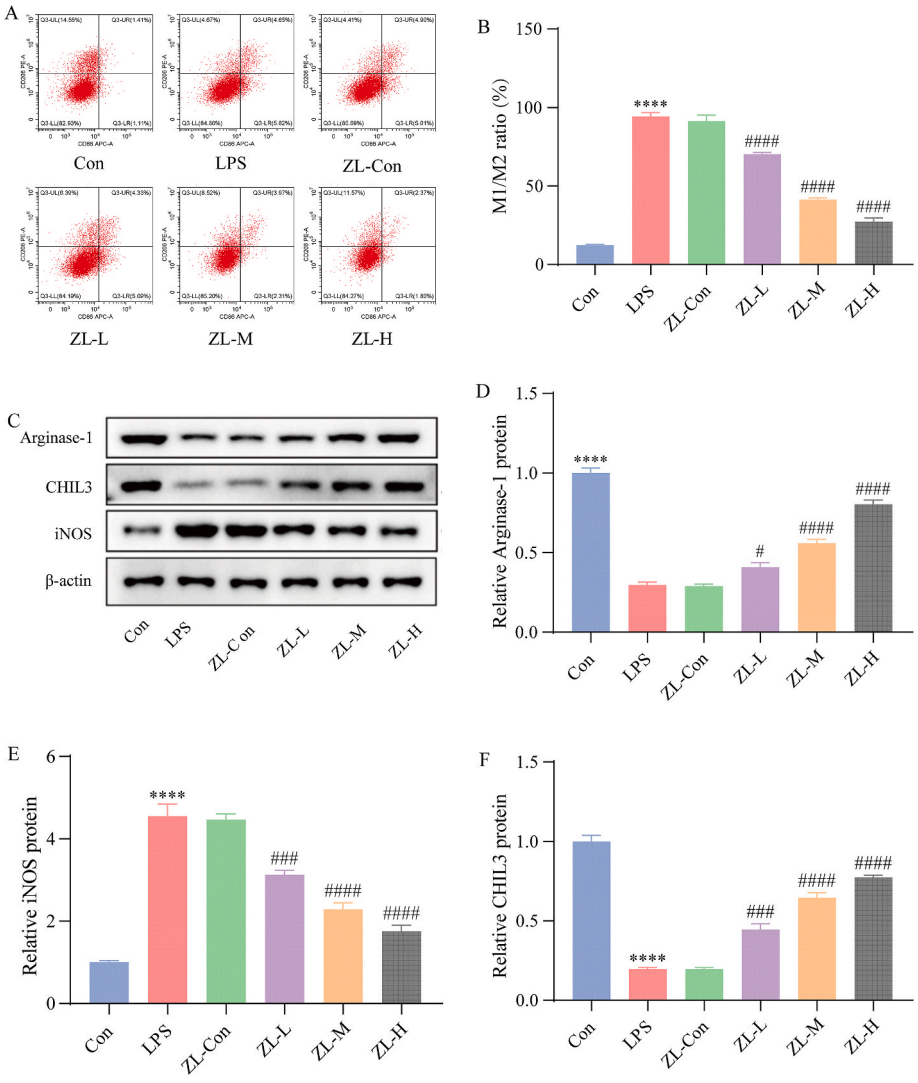

Fig. 2. ZL promoted macrophage polarization from M1 to M2. After LPS stimulation, RAW264.7 cells were treated with ZL serum. (A&B) Flow cytometry was performed to detect the M1/M2 ratio. (C ~ F) WB was used to measure the proteins expression, containing Arginase-1, CHIL3, and iNOS. The concentrations of ZL serum in the ZL-Con, ZL-L, ZL-M, and ZL-H groups were 0, 0.4, 0.8, and 1.6 g/kg/d, respectively. Mean ± standard error of mean was represented by bars. $^{****}P <$ 0.0001 compared with the Control group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with the LPS group.

(Fig. 3E–I). It suggested that ZL serum may target let-7i to inhibit the activation of the TLR9/MyD88 pathway.

3.4. ZL targeted let-7i to suppress inflammation

To verified the let-7i is the targets of ZL, let-7i inhibitor and ZL was applied after LPS stimulation. The expression of let-7i in the ZL group was significantly increased compared with that in the LPS group, and the addition of let-7i inhibitors significantly restored this effect (Fig. 4A). Similarly, the addition of let-7i inhibitors could restore the increase of IL-10 and TGF-β1 (Fig. 4B&C) as well as the decrease of TNF-αand IL-6 (Fig. 4D&E) after ZL intervention. These results demonstrated that ZL serum may target let-7i to suppressing inflammation in macrophages.

3.5. ZL targeted the let-7i/TLR9/MyD88 pathway to promote M2 polarization

On the basis of previous experiments, the effect of ZL serum on macrophage polarization was further explored. We also used classic biomarkers for macrophage sorting (Fig. 5A), and the results showed that ZL significantly inhibited the increase of the M1/M2 ratio caused by LPS, while the addition of let-7i inhibitors significantly restored this effect (Fig. 5B). Next, the mRNA and protein expressions of TLR9 and MyD88 were determined, and we found that ZL reduced the increased expressions of TLR9 and MyD88 caused by LPS while the addition of let-7i inhibitor significantly weakened the effects of ZL (Fig. 5C–G). As for the markers of macrophage polarization, iNOS showed a similar trend to the M1/M2 ratio, but Arginase-1 and CHIL3 expression showed an opposite station (Fig. 5H–K). It demonstrated that ZL may target let-7i to promote M2 polarization by regulating the TLR9/MyD88 signaling pathway.

4. Discussion

In this study, SD rats were orally administered with ZL to prepare the drug-containing serum, and different concentrations of ZL serum were used to treat LPS-stimulated mononuclear macrophages. The effects of ZL on macrophages were detected using ELISA, WB, and flow cytometry. After ZL intervention in macrophages, let-7i was inhibited, and the effects of the let-7i/TLR9/MyD88 pathway on macrophage polarization and inflammatory response were studied. The relationship between let-7i and TLR9 was verified by a dual luciferase reporter assay, providing a

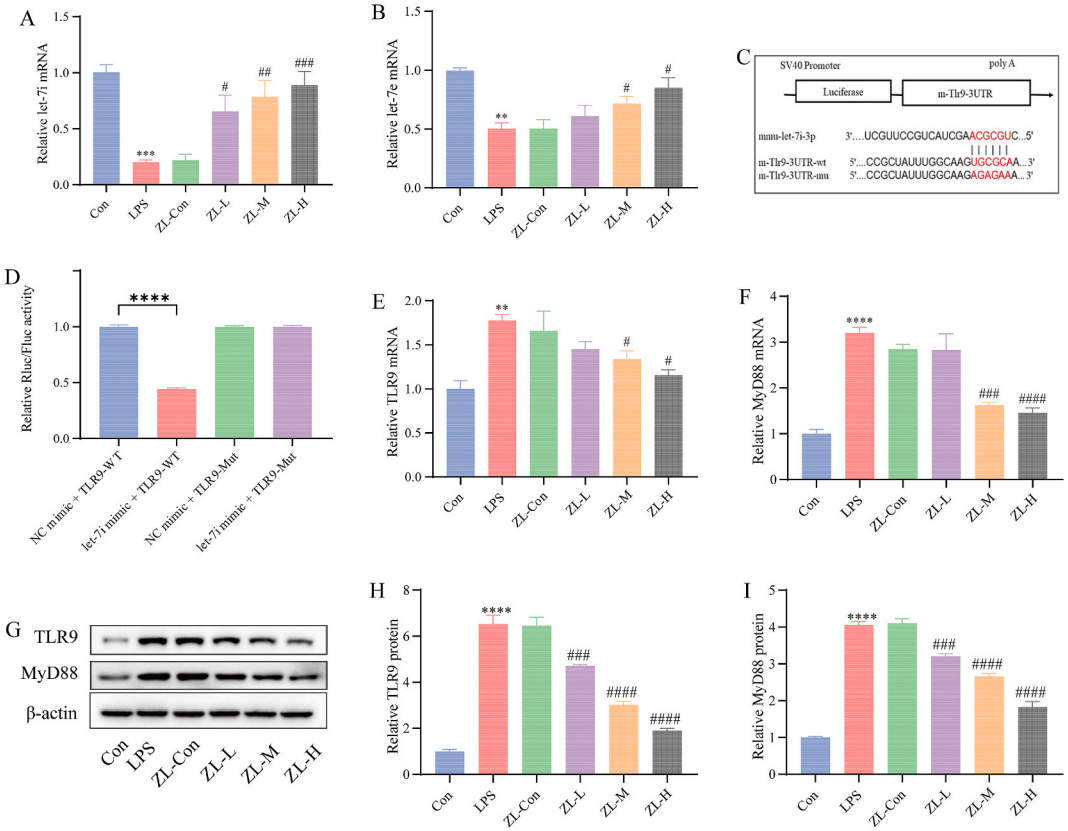

Fig. 3. ZL targeted let-7i to inhibiting the activation of the TLR9/MyD88 pathway. After LPS stimulation, RAW264.7 cells were treated with ZL serum. (A&B) The let-7i and let-7e expression were quantified by RT-qPCR. (C&D) Double luciferase was used to determine the relationship between let-7i and TLR9. (E&F) RT-qPCR was employed to quantify the mRNA expressions of TLR9 and MyD88. (G ~ I) WB was employed to quantify the protein expressions of TLR9 and MyD88. The concentrations of ZL serum in the ZL-Con, ZL-L, ZL-M, and ZL-H groups were 0, 0.4, 0.8, and 1.6 g/kg/d, respectively. Mean ± standard error of mean was represented by bars. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with the Control group unless noted otherwise, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with the LPS group.

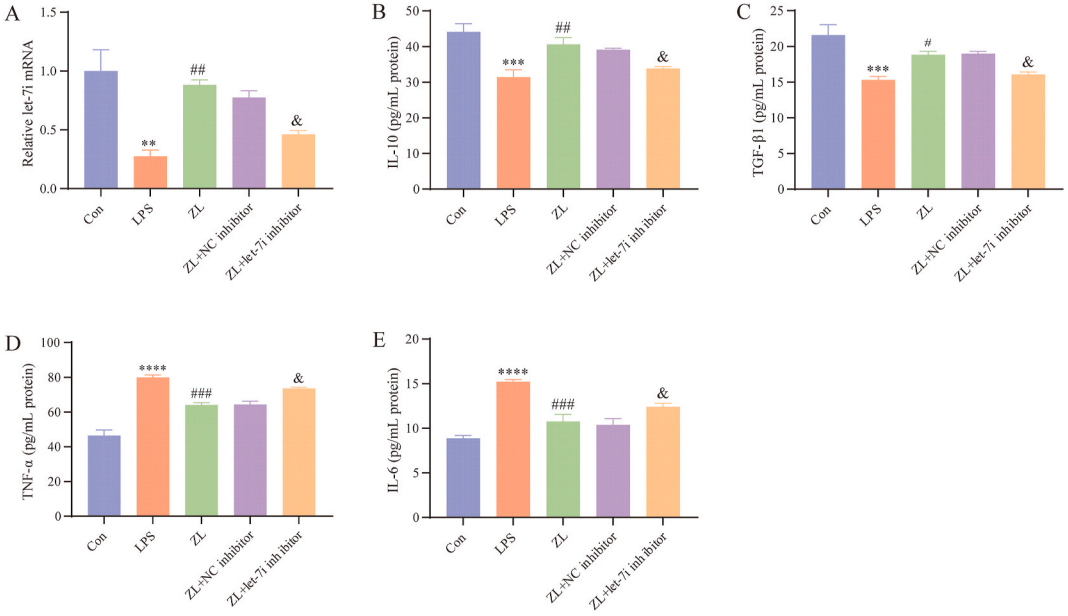

Fig. 4. ZL targeted let-7i to suppress inflammation. After LPS and ZL treatment, RAW264.7 cells were treated with NC inhibitor or let-7i inhibitor. (A) Let-7i expression was quantified by RT-qPCR. ELISA was performed to measure the contents of (B) IL-10, (C) TGF-β1, (D) TNF-α, and (E) IL-6. The concentration of ZL serum in the ZL group was 1.6 g/kg/d. Mean ± standard error of mean was represented by bars. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with the Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the LPS group, $^{\&}P < 0.05$, compared with the ZL group.

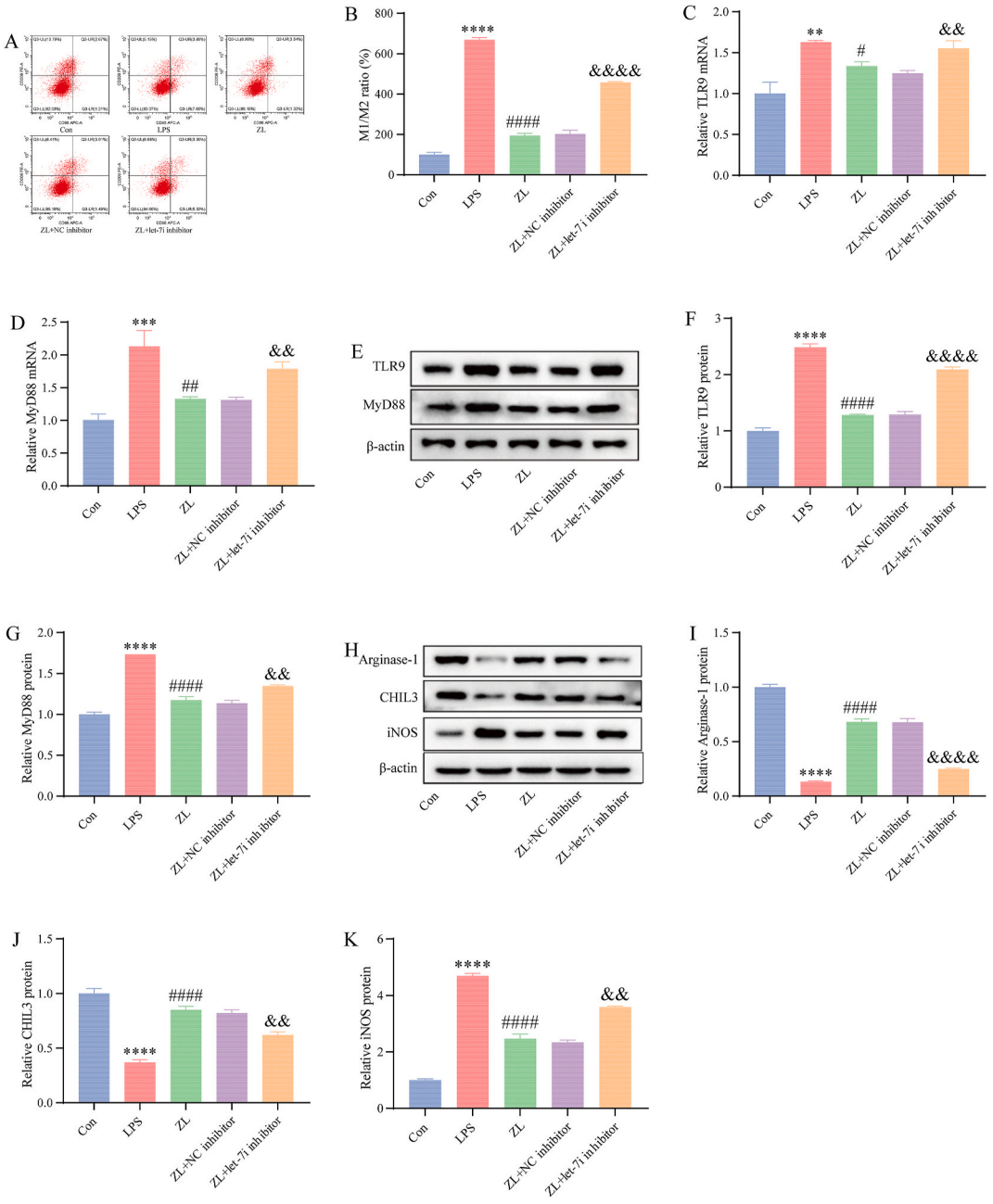

Fig. 5. ZL targeted the let-7i/TLR9/MyD88 pathway to promoting M2 polarization. After LPS and ZL serum treatment, RAW264.7 cells were treated with NC inhibitor or let-7i inhibitor. (A&B) Flow cytometry was performed to detect the M1/M2 ratio. (C ~ G) The expressions of MyD88 and TLR9 were determined by RT-qPCR and WB. (H ~ K) The expressions of Arginase-1, CHIL3 and iNOS were determined by WB. The concentration of ZL serum in the ZL group was 1.6 g/kg/d. Mean ± standard error of mean was represented by bars. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with the Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$, compared with the LPS group, $^{\&\&}P < 0.01$, $^{\&\&\&\&}P < 0.0001$, compared with the ZL group.

reference for the mechanism of action of ZL.

As reported, M1 macrophages can be identified by TLRs, CD25, and CD80, secreting pro-inflammatory cytokines such as IL-6, IL-12, and TNF-α. On the other hand, anti-inflammatory cytokines (IL-10 and TGF-β) are produced post-antigen presentation, acting on macrophages to promote M2 differentiation(Biswas and Mantovani, 2010; Martinez et al., 2013). IL-6 is distributed in the four biological processes and seven signaling pathways involved in the treatment of AIS by ZL(Li et al., 2022), indicating that IL-6 plays an important role in the treatment of AIS by ZL. In the present study, LPS was used to induce the inflammation of RAW264.7 cells, results manifested that ZL reduced the production of IL-6 and TNF-α, and increased the expressions of IL-10 and TGF-β. It is suggested that ZL may have an inhibitory effect on inflammation by regulating macrophage differentiation. CD86 and CD206 are two macrophage surface molecules that are used to label M1 and M2, respectively(Liamina et al., 2012). In addition, Arginase-1, CHIL3, and iNOS are the other three labels of macrophage polarization as functional modulators(Murray et al., 2014). Briefly, Arginase-1 and CHIL3 are linked with M2 polarization that promote tissue repair and anti-inflammatory responses. Among them, Arginase-1 is expressed by M2 macrophages as a key enzyme that metabolizes L-arginine to L-ornithine and urea(El Kasmi et al., 2008), and CHIL3 is linked with M2 macrophage activation. In addition, iNOS is involved in M1 polarization, thereby driving pro-inflammatory actions and hosting defense against pathogens(Mosser and Edwards, 2008). Our study indicated that ZL reversed LPS-induced the M1/M2 ratio, Arginase-1 and CHIL3 increase

as well as iNOS decrease, revealing ZL promotes macrophage polarization toward M2. However, the underlying molecular mechanism still unclear.

Previous studies indicated that ZL regulated microRNAs, including miR-30b-5p, miRNA-34b-3p, and miRNA-126-3p(Li et al., 2023; Liu et al., 2022). However, the impact of ZL on the let-7 family has not been reported yet. Evidences suggested that the let-7 family exerts diverse effects on macrophages in various diseases. Let-7b overexpression promotes M1 macrophage polarization in cancer(Chen et al., 2012). In contrast, let-7c-5p expression is reduced in a murine model of traumatic brain injury, and its overexpression encourages M2 macrophage polarization(Lv et al., 2018). In our study, we selected two let-7 family members, namely let-7i and let-7e, they both were downregulated under LPS stimulation. Among them, let-7i exhibited greater expression differences when treated with LPS and ZL. Consequently, we chose let-7i for subsequent research. As is widely known, LPS is a crucial molecule in activating the TLR/MyD88/NF-κB classical inflammatory pathway (Tang et al., 2005). In this study, dual-luciferase reporter assay validated that TLR9 was a target gene of let-7i. As a member of Toll-like receptors family which expressed on macrophages, TLR9 was reported that could recognize a series of pathogens and interact with MyD88, further regulating macrophage polarization(Behrens et al., 2011; Yasuda et al., 2005). Our research found that ZL significantly inhibited the activation of the TLR9/MyD88 signaling pathway. However, further validation is needed to determine whether ZL serum regulates macrophage polarization by targeting the let-7i/TLR9/MyD88 signaling pathway. We administered let-7i inhibitor simultaneously with ZL in LPS stimulated-cells to verify the adequacy and necessity of let-7i inhibitor in ZL for AIS. The cytokines, macrophage polarization, and relevant markers in the let-7i/TLR9/MyD88 pathway were subsequently measured and we found that let-7i inhibitor restored the effects of ZL. Considering the targeted interaction between let-7i and TLR9, we manifested that ZL upregulated the expression of let-7i and competitively inhibited the binding between TLR9 and MyD88 via let-7i, ultimately promoting M2 macrophage polarization and suppressing inflammation progression.

There are some limitations in our study. Firstly, we mainly focus on macrophage polarization, so we only conducted relevant research on macrophages. Our previously published article has confirmed that ZL can effectively alleviate cerebral infarction area and pathological changes, neurological deficit score, cerebral hemoglobin content, and blood-brain barrier permeability in AIS model rats(Geng et al., 2023), and we here prepared ZL serum that can effectively simulate the state of ZL *in vivo*, these efforts could to some extent supplement the lack of animal experiments in this study. Finally, this study can only provide preclinical evidence despite our rigorous design, and we will further verify the effect of ZL on the let-7i/TLR9/MyD88 pathway through clinical trials in the future.

In conclusion, ZL upregulated let-7i expression, and the high level of let-7i targeted TLR9, competitively inhibiting TLR9 and subsequently suppressing the activation of the TLR9/MyD88 pathway. This promoted macrophage transition to the M2 phenotype, inhibited pro-inflammatory cytokines (IL-6 and TNF-α) expression, and enhanced anti-inflammatory cytokines (IL-10 and TGF-β) expression. The anti-inflammatory effects of M2 macrophages provide neuroprotection. Therefore, it was demonstrated that ZL has a promising efficacy in controlling secondary inflammatory responses in AIS, and the clinical application of ZL holds significance for favorable AIS prognosis.

CRediT authorship contribution statement

Ya-Fei Kang: Writing – original draft, Resources, Formal analysis, Data curation. **Xue Bai:** Writing – original draft, Validation, Methodology, Formal analysis. **Kong-Yu Wang:** Visualization, Validation, Methodology. **Tao Wang:** Software, Methodology, Formal analysis. **Chuan-Ling Pan:** Visualization, Formal analysis, Data curation. **Cheng Xie:** Software, Methodology, Formal analysis. **Bo Liang:** Writing – review & editing, Validation, Supervision, Investigation. **Hui-Ling Liao:** Writing – review & editing, Visualization, Supervision, Resources.

Declaration of competing interest

None.

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