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ORIGINAL ARTICLE

Schisandrin B Attenuates Inflammation in LPS-Induced Sepsis Through miR-17-5p Downregulating TLR4

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Abstract— To investigate the mechanism of *Schisandrin B* (*Sch B*) on the inflammation in LPS-induced sepsis. Sepsis mouse model was established by injecting LPS. qRT-PCR and western blot were used to measure the expression of miR-17-5p and TLR4. ELISA was used to test the concentrations of IL-1β and TNF-α. *Sch B* could increase miR-17-5p expression, promote inflammation, and decrease TLR4 expression in sepsis mice and LPS-induced macrophages. Moreover, miR-17-5p could negatively regulate TLR4. Overexpression of miR-17-5p suppressed the concentrations of inflammatory factors (IL-1β and TNF-α) in LPS induced-macrophages, while pcDNA-TLR4 could change the inhibition effect. Additionally, miR-17-5p inhibitor changed the inhibitory effects of *Sch B* on TLR4 expression and the concentrations of IL-1β and TNF-α in LPS induced-macrophages. *Sch B* could attenuate inflammation in LPS-induced sepsis through miR-17-5p downregulating TLR4.

KEY WORDS: sepsis; *Schisandrin B*; miR-17-5p; TLR4; inflammation.

INTRODUCTION

Sepsis is a systemic inflammatory syndrome caused by infection, which leads to multiple organ failures [1]. The mortality rate of patients with sepsis is very high [2]. It shows that excessive inflammatory response is one of its pathological characteristics [3]. However, there is no effective treatment for the inflammation in sepsis in clinic.

Toll-like receptor 4 (TLR4) is a member of TLR family, and its activation by lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is responsible for acute and chronic inflammatory disorders [4]. It has been reported that TLR4 exerts important functions in sepsis. For example, Venancio *et al.* [5] showed that CETP attenuated the inflammatory response in LPS-induced sepsis through reducing TLR4 expression. Therefore, modulation of TLR4 is a potential strategy to treat the inflammation in sepsis.

Schisandrin B (*Sch B*) is a dibenzocyclooctadiene lignan isolated from *Schisandra chinensis*, a fruit of the Chinese magnolia vine [6]. The structure of *Sch B* is shown in Fig. 1a [7]. *Sch B* has been reported to exhibit antitumor, antioxidative, and anti-inflammatory properties. Previous *in vitro* and *in vivo* studies have demonstrated that *Sch B* can attenuate inflammation in hind limb I/R skeletal muscle injury through MAPK/NF-κB pathways in rats [8] and reduce inflammatory response induced by traumatic spinal cord injury *via* p53 signaling pathway

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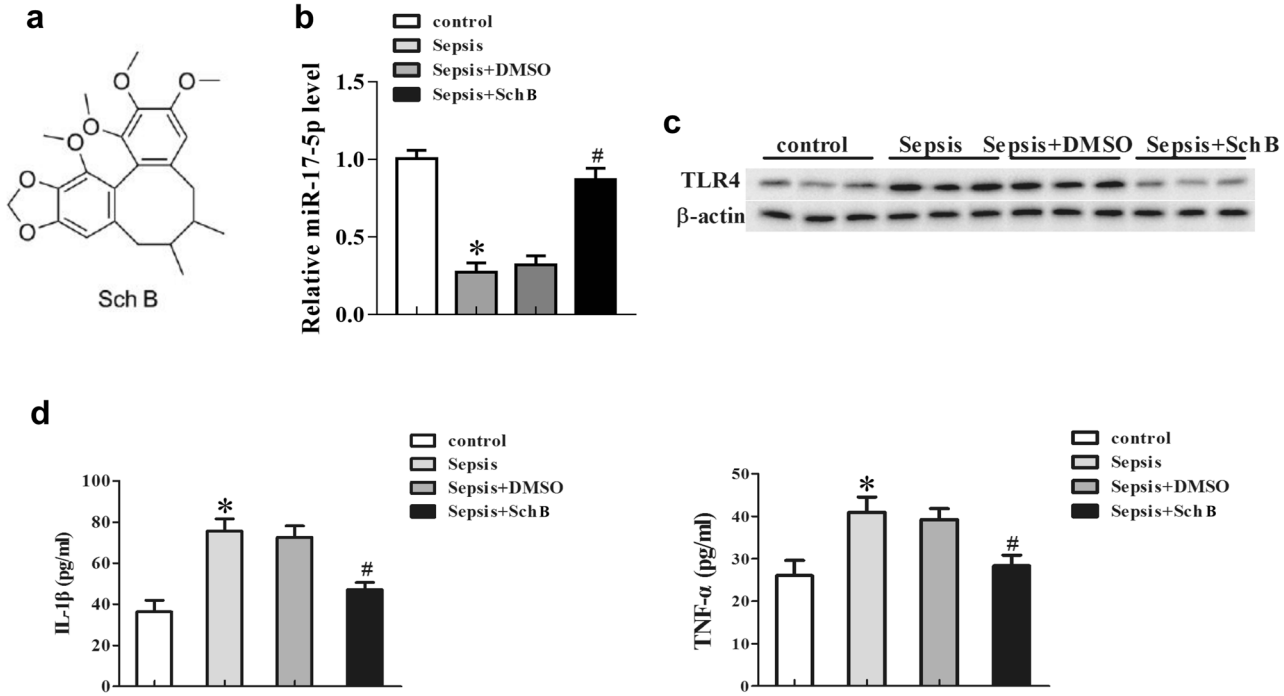

Fig. 1. *Sch B* increased miR-17-5p and inflammation, and decreased TLR4 in sepsis mice. Mice were pre-treated with 40 mg/kg *Sch B* for 1 h and then injected with 50 mg/kg LPS to induce sepsis. Four hours later, PB was collected from mice and PBMCs were isolated for qRT-PCR, western blot, and ELISA. **a** The structure of *Sch B*. **b** MiR-17-5p expression. **c** TLR4 expression. **d** The concentrations of inflammatory factors (IL-1β and TNF-α). $^{*}P < 0.01$ *vs* control group; $^{\#}P < 0.01$ *vs* sepsis + DMSO group.

[6]. In addition, *Sch B* could exert anti-inflammatory effect in LPS-induced sepsis through TLR4 pathway [9], which indicated that *Sch B* was closely related to the inflammatory response in sepsis. However, the effects and mechanisms of *Sch B* on the inflammation in sepsis remain inadequately understood.

MicroRNAs (miRNAs), a class of endogenous noncoding RNA with the length of 21–25 nucleotides [10], have considered as key regulators of inflammatory response and potential biomarkers for sepsis [11]. MiR-17-5p is an oncogene in various cancers, such as malignant pleural mesothelioma [12], breast cancer [13], and pancreatic cancer [14]. Acosta-Herrera *et al.* [15] revealed that miR-17-5p might be related to sepsis-induced acute lung injury. However, the role of miR-17-5p in sepsis has not been reported.

Based on the biological prediction (TargetScan.org), miR-17-5p could target TLR4. Thus, we hypothesized that miR-17-5p might be involved in the inflammation of sepsis through TLR4. Hence, the aim of the current study was to investigate the effect of miR-17-5p/TLR4 on the inflammatory response induced by LPS and explore the mechanism of *Sch B* on the inflammation in LPS-induced sepsis.

METHODS

Sepsis Mouse Model

Female C57BL/6 mice were purchased from the Shanghai Lab. Animal research center. Mice were received LPS (50 mg/kg body weight) by intraperitoneal injection to induce experimental sepsis. In the control group, mice were received the same amount of PBS by intraperitoneal injection. In sepsis + Sch B group, mice were received *Sch B* 40 mg/kg body weight by intraperitoneal injection before 1 h of the LPS injection [9]. Mice in sepsis + DMSO group were given the same amount of DMSO before 1 h of the LPS injection.

The Isolation of Peripheral Blood Mononuclear Cells

Four hours after LPS injection, the peripheral blood (PB) of mice from each group was collected in anticoagulant tubes, and the peripheral blood mononuclear cells (PBMCs) were separated by Ficoll density gradient centrifugation. After washing in PBS for three times, PBMCs were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, USA) at

37 °C in in a humidified atmosphere of 5% CO_2. Cell viability was checked using trypan blue staining, and more than 95% of the cells were viable [16].

Cell Culture

The murine macrophage cell line (RAW264.7) was obtained from the Cell Bank of Shanghai Academy of Science. RAW264.7 cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell Grouping and Processing

The grouping method of RAW264.7 cells is as follows: (1) control group: normal RAW264.7 cells. (2) LPS group: cells were treated with 1 μg/ml LPS for 30 min. (3) LPS + Sch B group: cells were treated with 16 μM *Sch B* for 1 h [9] and then treated with 1 μg/ml LPS for 30 min. (4) LPS + miR-17-5p mimic group: cells were transfected with miR-17-5p mimic for 24 h using Lipofectamine 2000 (Invitrogen) and then treated with 1 μg/ml LPS for 30 min. (5) LPS + miR-17-5p mimic + pcDNA-TLR4 group: cells were transfected with miR-17-5p mimic and pcDNA-TLR4 for 24 h using Lipofectamine 2000 and then treated with 1 μg/ml LPS for 30 min. (6) LPS + Sch B + miR-17-5p inhibitor group: cells were transfected with miR-17-5p inhibitor for 24 h using Lipofectamine 2000 and then treated with 16 μM *Sch B* for 1 h and 1 μg/ml LPS for 30 min. MiR-17-5p mimic, miR-17-5p inhibitor, pcDNA-TLR4, and their corresponding controls were purchased from GenePharma (Shanghai, China).

Quantitative Real-Time PCR

Trizol reagent (Invitrogen) was used for total RNA extraction from cells following the manufacturer's protocols. Then, cDNAs were synthesized, and PCR reaction was performed using ABI Prism 5700 Sequence Detection System (SDS; Applied Biosystems, Foster City, CA, USA). Results were analyzed using ABI Prism 5700 SDS software (Applied Biosystems). The comparative method $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of miR-17-5p.

Western Blot

Total protein of cells was extracted with RIPA Lysis buffer (Beyotime, China) according to the manufacturer's instructions. The concentrations of proteins were detected by BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples that contained same amount of extracted proteins were separated on 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). The membranes were blocked with 5% skim milk at room temperature for 1 h and added with primary antibody TLR4 (1:500, Abcam, Chicago, IL, USA), β-actin (1:1000, Abcam), for overnight incubation at 4 °C. After washing with Tris-buffered saline tween for three times, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (1:1000, Abcam) at room temperature for 1 h. Blots were developed using an ECL chemiluminescence substrate (Thermo Fisher Scientific) and analyzed by Image J software.

Cytokine Measurement

The concentrations of IL-1β and TNF-α in serum from mice with different treatment and RAW264.7 cell culture supernatant were measured by enzyme-linked immunosorbent (ELISA) kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.) following the manufacturer's instructions.

Dual-Luciferase Reporter Assay

In order to testify the targeting relationship between miR-17-5p and TLR4, TLR4 3′UTR fragments with wild type (WT) or mutant (Mut) miR-17-5p putative binding sites were cloned by PCR. PCR products were inserted into the pMir-Glo vector (synthesized by Shanghai GenePharma Co., Ltd.). HEK293 cells were seeded in 96-well plate at a density of 1×10^5 cells/well. Then, miR-17-5p mimics, miR-17-5p inhibitor, and their corresponding controls (pre-NC, NC) were co-transfected with luciferase reporter vector into HEK293 cells using Lipofectamine 2000, respectively. Twenty-four hours after transfection, Firefly and Renilla luciferase activities were sequentially measured using the Dual Glo™ Luciferase Assay system (Promega).

Statistical Analysis

The SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) was applied to analyze data, and all results were expressed as mean ± standard deviation (SD). The comparison between two groups was analyzed by Student's *t* test. The comparison of measured data among groups was

assessed using one-way ANOVA. A significant difference was set at $P < 0.05$.

RESULTS

Sch B Increased miR-17-5p and Inflammation and Decreased TLR4 in Sepsis Mice

Initially, we explored whether *Sch B* could affect the expression of miR-17-5p and TLR4 as well as inflammation in sepsis mice. Mice were pre-treated with 40 mg/kg *Sch B* for 1 h and then injected with 50 mg/kg LPS to induce sepsis. Four hours later, PB was collected from mice, and PBMCs were isolated for qRT-PCR, western blot, and ELISA. The structure of *Sch B* is shown in Fig. 1a. Results showed that miR-17-5p expression significantly decreased, and TLR4 expression markedly increased in the sepsis group, while *Sch B* treatment could change these expression trends (Fig. 1b, c). In addition, *Sch B* treatment could inhibit the increased concentrations of inflammatory factors (IL-1β and TNF-α, Fig. 1d) induced by sepsis.

Sch B Increased miR-17-5p and Decreased TLR4 in Macrophages Induced by LPS

To investigate the effect of *Sch B* on the expression of miR-17-5p and TLR4 as well as inflammation in macrophages induced by LPS, RAW264.7 cells were treated with 16 μM *Sch B* for 1 h before LPS treatment. Compared with the control group, LPS could downregulate miR-17-5p expression, while *Sch B* treatment could reverse the effect (Fig. 2a). In addition, compared with the LPS group, *Sch B* could inhibit the protein expression of TLR4 (Fig. 2b). The concentrations of inflammatory factors (IL-1β and TNF-α) in the LPS + Sch B group were lower than that in the LPS group (Fig. 2c), which coincided with the results in animal experiments.

Overexpression of miR-17-5p Inhibited Inflammation in Macrophages Induced by LPS

RAW264.7 cells were divided into four groups (control group, LPS group, LPS + pre-NC group, and LPS + miR-17-5p mimic group) to explore the role of miR-17-5p on inflammation in macrophages. MiR-17-5p mimic was used to increase the expression of miR-17-5p in RAW264.7 cells (Fig. 3a). Our results suggested that LPS upregulated the concentrations of inflammatory factors (IL-1β and TNF-α) in RAW264.7 cells (Fig. 3b), whereas miR-17-5p mimic treatment removed the effect of LPS.

MiR-17-5p Directly Targeted TLR4

Based on the biological prediction (TargetScan.org), miR-17-5p could target TLR4 (Fig. 4a). Therefore, the dual-luciferase reporter assay was used to verify whether miR-17-5p targeted TLR4. Results showed that the luciferase activity of TLR4-WT in miR-17-5p mimic group was significantly decreased, and the luciferase activity of TLR4-WT in miR-17-5p inhibitor group was significantly increased (Fig. 4b). However, the luciferase activity of TLR4-Mut in both miR-17-5p mimic group and miR-17-5p inhibitor group had no significant differences (Fig. 4b). In addition, overexpression of miR-17-5p could reduce the mRNA and protein expression of TLR4, while knockdown of miR-17-5p could enhance the mRNA and protein expression of TLR4 (Fig. 4c, d).

Overexpression of miR-17-5p Inhibited Inflammation in LPS-Induced Macrophages by Downregulating TLR4

In order to analyze the mechanisms of miR-17-5p/TLR4 in inflammation of macrophages, their expressions were measured in transfected cells *in vitro* using qRT-PCR and western blot, and the concentrations of inflammatory factors (IL-1β and TNF-α) were detected by ELISA. Our results suggested that LPS decreased miR-17-5p expression (Fig. 5a) and increased TLR4 expression (Fig. 5b), while miR-17-5p mimic reversed the trends. Meanwhile, pcDNA-TLR4 treatment removed the decreased TLR4 expression caused by miR-17-5p mimic (Fig. 5b). Moreover, ELISA results showed that overexpression of miR-17-5p suppressed the concentrations of IL-1β and TNF-α in LPS-induced RAW264.7 cells (Fig. 5c). In contrary, pcDNA-TLR4 promoted the expression of inflammatory factors (Fig. 5c).

Sch B Inhibited Inflammation in LPS-Induced Macrophages through miR-17-5p Downregulating TLR4

Lastly, we verified the modulatory role of *Sch B* on inflammation in LPS-induced macrophages. RAW264.7 cells were transfected with miR-17-5p inhibitor and treated with *Sch B* for 1 h and then treated with LPS for 30 min. As shown in Fig. 6a, b, *Sch B* could upregulate miR-17-5p expression and downregulate TLR4 expression, while miR-17-5p inhibitor reversed the effects. Meanwhile, miR-17-5p inhibitor changed the inhibitory effects of *Sch B* on the concentrations of inflammatory factors (IL-1β and TNF-α) in LPS-induced macrophages (Fig. 6c).

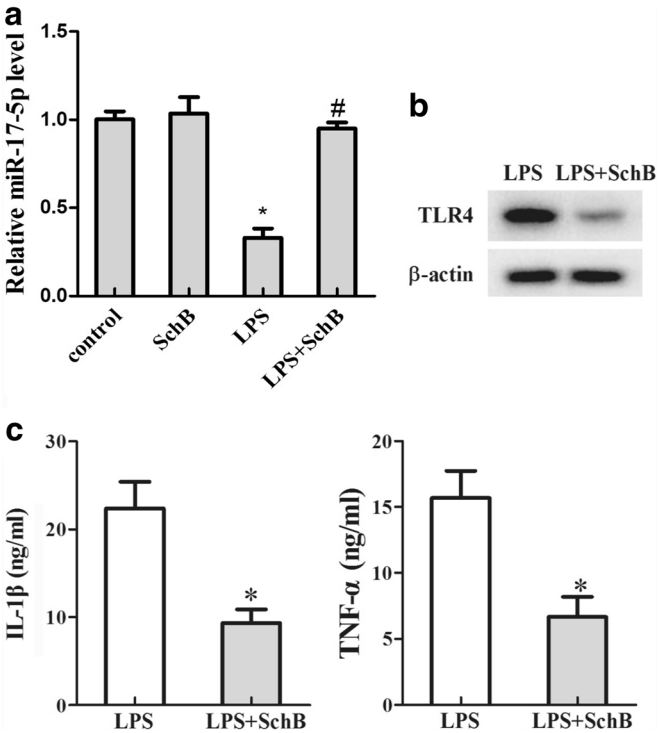

Fig. 2. *Sch B* increased miR-17-5p and decreased TLR4 in macrophages induced by LPS. RAW264.7 cells were divided into control group, Sch B group, LPS group, and LPS + Sch B group. Cells were treated with 16 μM *Sch B* for 1 h before LPS treatment. **a** MiR-17-5p expression. **b** TLR4 expression. **c** The concentrations of inflammatory factors (IL-1β and TNF-α). $^{*}P < 0.01$ *vs* LPS group.

DISCUSSION

Schisandra chinensis is a traditional herbal medicine in China, which has the pharmacological effects of anti-oxidation, immune regulation, anti-tumor, anti-virus, and bacteriostasis [17]. *Sch B* is a main active component of *Schisandra chinensis* and plays an important role in inflammation [18]. For example, Liu *et al*. [19] revealed that *Sch B* could inhibit LPS-induced inflammatory response in microglia through activating PPAR-γ. In sepsis, Xu *et al*. [9] demonstrated that *Sch B* might be a novel anti-inflammatory candidate drug for treating sepsis. However, little studies report the mechanism of *Sch B* in inflammation in sepsis.

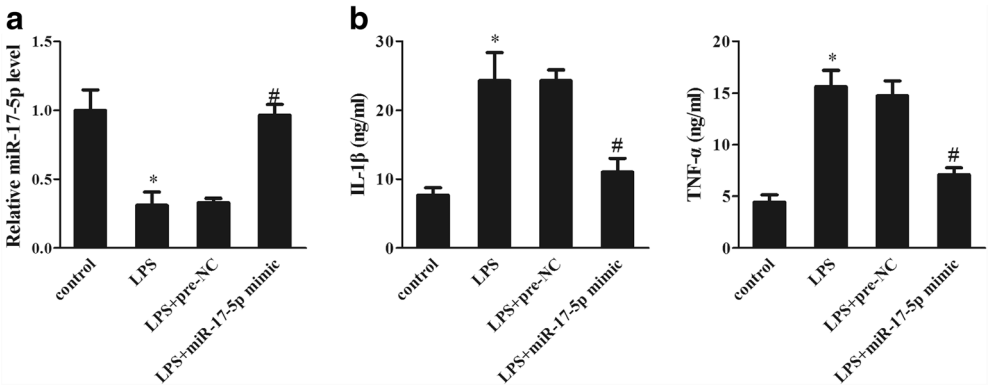

Fig. 3. Overexpression of miR-17-5p inhibited inflammation in macrophages induced by LPS. RAW264.7 cells were divided into control group, LPS group, LPS + re-NC group, and LPS + miR-17-5p mimic group. **a** MiR-17-5p expression. **b** The concentrations of inflammatory factors (IL-1β and TNF-α). $^{*}P < 0.01$ *vs* control group; $^{\#}P < 0.01$ *vs* LPS + pre-NC group.

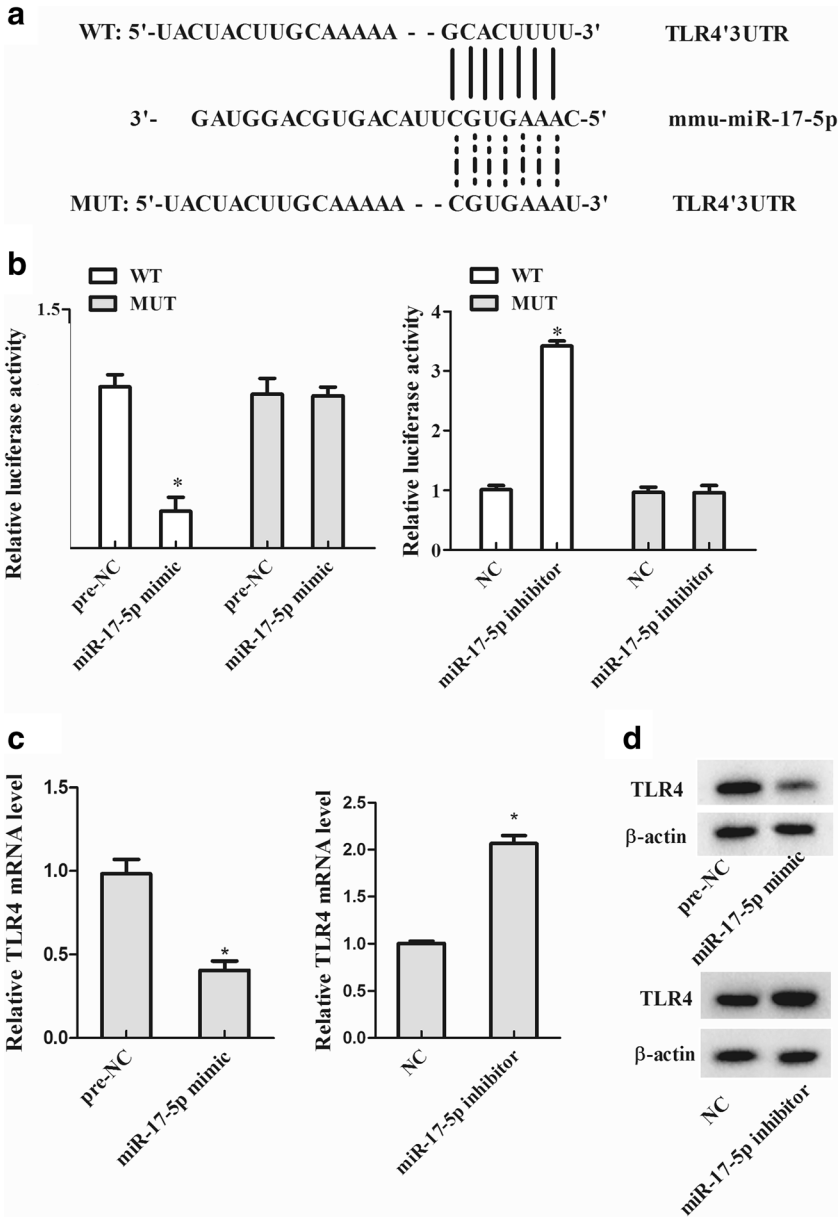

Fig. 4. MiR-17-5p directly targeted TLR4. **a** The bind sites between miR-17-5p and TLR4. **b** The luciferase activity of TLR4. **c** TLR4 mRNA expression in RAW264.7 cells transfected with miR-17-5p mimic or miR-17-5p inhibitor. **d** TLR4 protein expression in RAW264.7 cells transfected with miR-17-5p mimic or miR-17-5p inhibitor. *$P < 0.01$ *vs* pre-NC or NC.

As reported previously, LPS is widely used to activate macrophages in cell and animal models for evaluating potential anti-inflammatory drugs [20], and the most commonly used model of sepsis is the immune response induced by LPS [21]. It has been showed that TLR4 is the main receptor for recognizing LPS [22]. After stimulation by LPS, the TLR4 signal pathway will be activated in macrophages and then caused immune cells to release pro-inflammatory cytokines (IL-1β, TNF-α, *etc.*) [23]. These cytokines will produce systemic inflammatory response, which is the characteristic of early sepsis [21]. In this study, we measured the effect of *Sch B* on inflammation in the LPS-induced sepsis mouse model and macrophage. We found that *Sch B* inhibited TLR4 expression and reduced the concentration of both IL-1β and

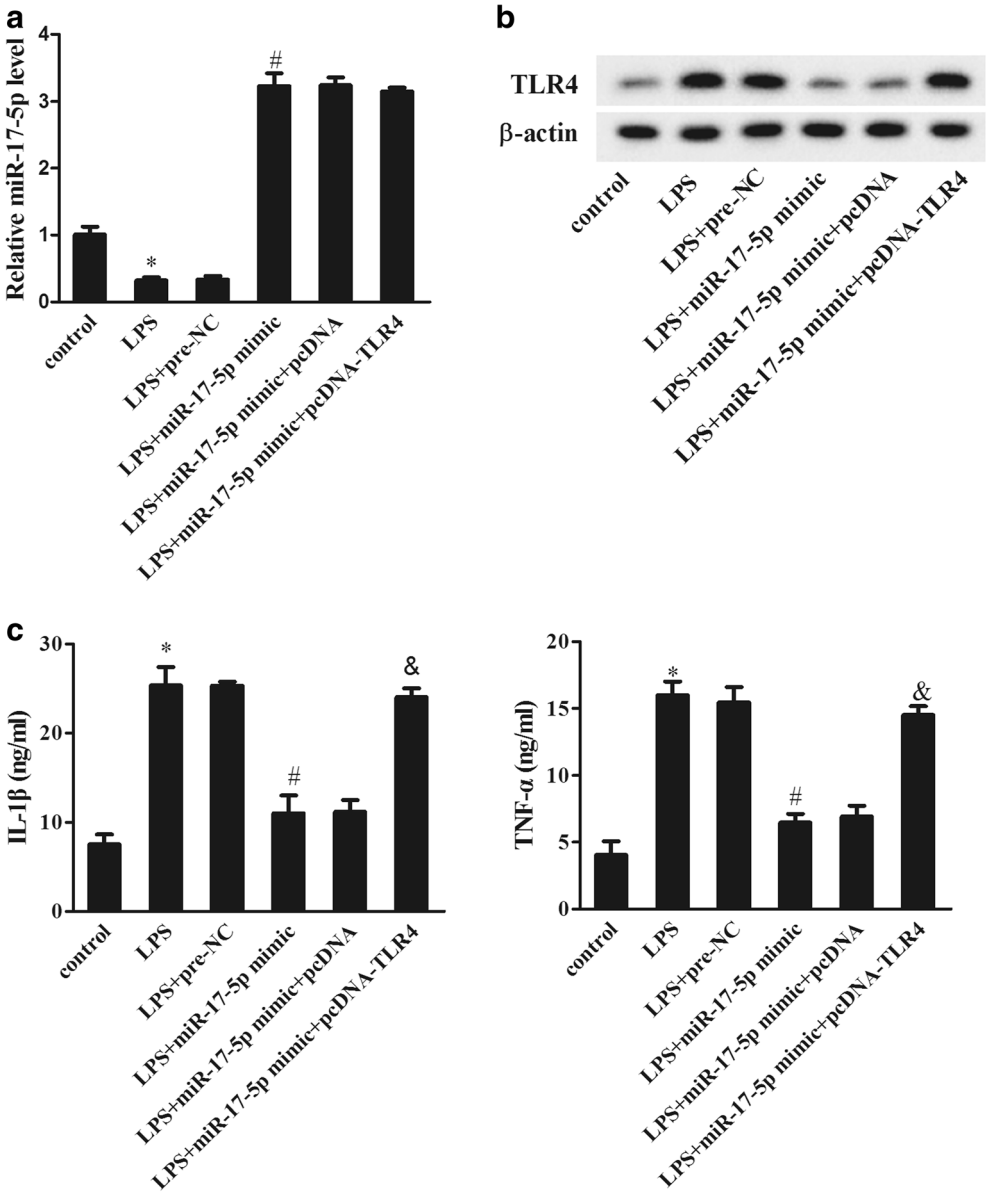

Fig. 5. Overexpression of miR-17-5p inhibited inflammation in LPS-induced macrophages by downregulating TLR4. RAW264.7 cells were divided into six groups, namely control group, LPS group, LPS + pre-NC group, LPS + miR-17-5p mimic group, LPS + miR-17-5p mimic + pcDNA group, and LPS + miR-17-5p mimic + pcDNA-TLR4 group. **a** MiR-17-5p expression. **b** TLR4 expression. **c** The concentrations of inflammatory factors (IL-1β and TNF-α). *$P < 0.01$ *vs* control group; $^{\#}P < 0.01$ *vs* LPS + pre-NC group; and $P < 0.01$ *vs* LPS + miR-17-5p mimic + pcDNA group.

TNF-α in sepsis mice and LPS-induced macrophage, which indicated that the anti-inflammatory effect of *Sch B* in sepsis was related with TLR4. The results were consistent with previous study [9], but how does *Sch B* regulate TLR4?

In the present study, we found that miR-17-5p was lowly expressed in sepsis mice and LPS-induced macrophage, which suggested a relationship between miR-17-5p and sepsis. In addition, *Sch B* could upregulate miR-17-5p expression in sepsis mice and LPS-induced macrophage. MiR-17-5p is a member of miR-17-92 cluster, which is involved in various diseases [24]. Moreover, miR-17-5p can also participate in the regulation of inflammatory response. Coucha *et al*.

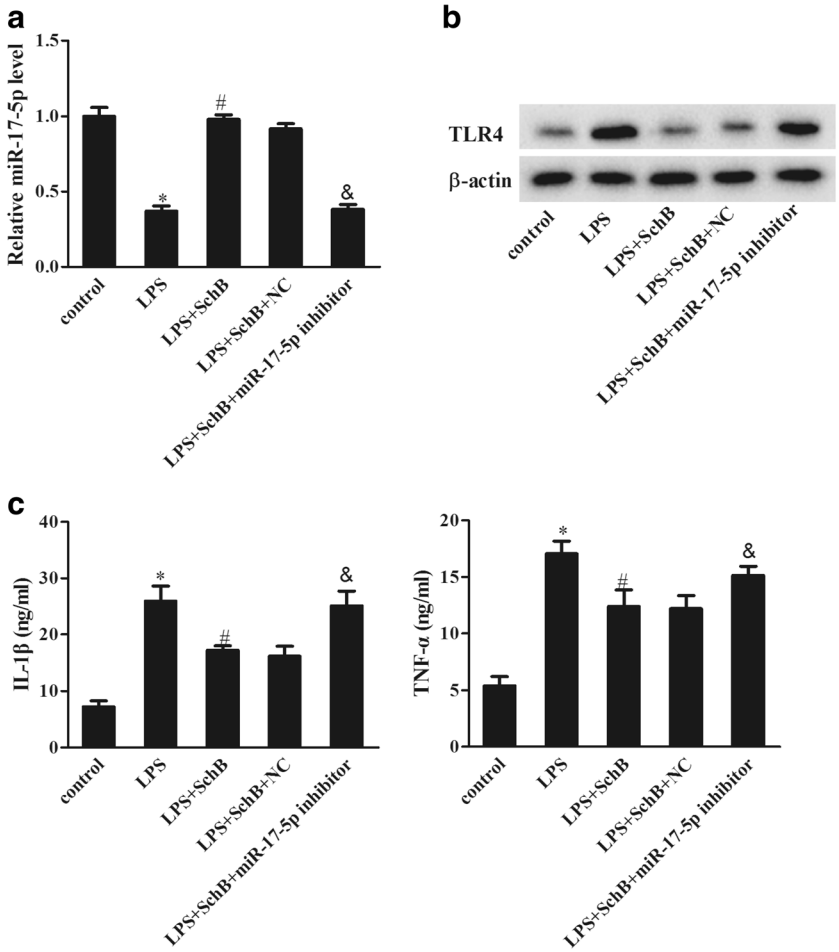

Fig. 6. *Sch B* inhibited inflammation in LPS-induced macrophages through miR-17-5p downregulating TLR4. RAW264.7 cells were transfected with miR-17-5p inhibitor and treated with *Sch B* for 1 h and then treated with LPS for 30 min. **a** MiR-17-5p expression. **b** TLR4 expression. **c** The concentrations of inflammatory factors (IL-1β and TNF-α). $^{*}P < 0.01$ *vs* control group; $^{\#}P < 0.01$ *vs* LPS group; and $P < 0.01$ *vs* LPS + SchB + NC group.

[25] showed that the decreased expression of miR-17-5p is related to high-fat diet–induced retinal inflammation. Chen *et al.* [26] revealed that IRE1-α inhibition decreased inflammasome activation in rats after neonatal hypoxia-ischemic brain injury through miR-17-5p. In our study, we showed that miR-17-5p could negatively regulate TLR4 expression, and overexpression of miR-17-5p could suppress the inflammation in LPS-induced macrophage *via* downregulating TLR4. Hence, we hypothesized that the effect of *Sch B* on TLR4 might be related with miR-17-5p. As expected, the further study proved this hypothesis.

In conclusion, we provided evidence that *Sch B* could alleviate inflammation in LPS-induced macrophages through miR-17-5p downregulating TLR4. These findings elucidated a novel molecular mechanism of *Sch B* in the inflammation of sepsis.

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