Forsythoside A protects against lipopolysaccharide-induced acute lung injury through up-regulating microRNA-124

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Abstract

Acute lung injury (ALI) is a life-threatening disease without effective pharmacotherapies, so far. *Forsythia suspensa* is frequently used in the treatment of lung infection in traditional Chinese medicine. In searching for natural anti-inflammatory components, the activity and the underlying mechanism of Forsythoside A (FA) from *Forsythia suspensa* were explored. In this paper, BALB/c mice and murine RAW 264.7 cells were stimulated by LPS to establish inflammation models. Data showed that FA inhibited the production of TNF-α and IL-6 and the activation of STAT3 in LPS-stimulated RAW 264.7 cells. Additionally, FA increased the expression level of miRNA-124 (miR-124). Furthermore, the inhibitory effect of FA on STAT3 was counteracted by the treatment of miR-124 inhibitor. Critically, FA ameliorated LPS-induced ALI pathological damage, the increase of lung water content and inflammatory cytokine, cells infiltration and activation of the STAT3 signaling pathway in BALB/c mice. Meanwhile, FA up-regulated the expression of miR-124 in lungs, while administration with miR-124 inhibitor attenuated the protective effects of FA. Our results indicated that FA alleviates LPS-induced inflammation through up-regulating miR-124 *in vitro and in vivo*. These findings indicate the potential of FA and miR-124 in the treatment of ALI.
Introduction

Acute lung injury (ALI) is a life-threatening disease with severe inflammatory responses. In clinic, ALI is characterized by pulmonary inflammation, increased vascular permeability, acute onset, and hypoxemia [1]. The currently available therapies include conservative fluid strategy, lung-protective ventilation, prone positioning and high-frequency oscillatory ventilation. Although advance has been made in treatment modalities, the mortality rate in patients with ALI is still 36 – 44% in the past decades [2].

Recent studies suggest that numerous microRNAs (miRNAs) play a vital role in the progress of inflammatory responses in ALI [3]. It has found that miR-7 deficiency up-regulated the expression of Kruppel-like factor 4 in lung tissues, thereby modulating the NF-κB, AKT, and ERK pathways to attenuate inflammation in ALI [4]. Moreover, the deficiency of miR-155 inhibited the inflammatory responses to protect mice from LPS-induced ALI [5]. Besides, the inhibition of miR-429 induced the increase of dual-specificity phosphatase 1, which inactivated the p38 MAPK to decrease pro-inflammatory cytokines in ALI [6]. These findings indicate that miRNA may be a new therapeutic strategy against inflammatory lung diseases.

Forsythia suspensa (Lianqiao), a famous traditional Chinese medicine, is extensively used in the treatment of pneumonia in clinic for centuries due to its significant anti-inflammatory activity [7, 8]. Recent research has demonstrated that the ethanol extract of Forsythia suspensa, markedly suppresses influenza A virus-induced regulated on activation normal T cell expressed and secreted secretion by human bronchial epithelial cells, inhibits replication of influenza viruses in mice lung tissue, and relieves lung tissue lesion [9, 10]. The aqueous extract of Forsythia suspensa ameliorates inflammatory responses by activating NF-E2-related factor 2 (Nrf2) and suppressing
NF-κB in RAW 264.7 cells induced by LPS or TNF-α [11]. Considered the clinical and experimental evidence, it is potential to identify a lead compound from Forsythia suspensa to treat ALI. Previous research reported that forsythiside A (FA), a natural product from Forsythia suspensa, inhibited zymosan-induced peritonitis and S. aureus-stimulated inflammatory responses through attenuating the NF-κB pathway [12, 13]. Therefore, we focused on the anti-ALI properties of FA.

In this study, we first demonstrate that FA exerts significant anti-inflammatory effects in vitro and in vivo. Mechanism study demonstrated that this compound up-regulates miR-124, and subsequently inhibits STAT3 activity. These findings suggested that FA could be a promising agent in the treatment of ALI and supported the critical role of miRNAs in suppressing inflammatory responses.

Materials and methods

Reagents

FA (purity > 98%) was purchased from Toronto Research Chemicals (Toronto, Canada). DMEM, FBS, penicillin/streptomycin, Alexa Fluor 488-conjugated anti-Rabbit IgG antibody, BCA protein assay kit, ECL kit were obtained from Thermo Fisher Scientific (Waltham, USA). IL-6 and TNF-α ELISA kits were obtained from Dakewe (Beijing, China). Paraformaldehyde was purchased from Leica (Heerbrugg, Switzerland). BSA was purchased from Merck (Darmstadt, Germany). Antibodies against STAT3 (cat. no. 12640), phospho-STAT3 (Tyr705, cat. no. 9145) and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, USA). DAPI was obtained from Bioss (Beijing, China). β-actin antibody
(cat. no. BM5422) was purchased from Boster (Wuhan, China). PVDF membranes was obtained from Bio-Rad (Hercules, USA). MiRcute miRNA Kit was purchased from TIANGEN (Beijing, China). PrimeScript™ RT reagent Kit with gDNA Eraser and SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) were obtained from Takara (Kusatsu, Japan). The miR-124 mimic, miR-124 inhibitor, riboFECT™ CP Transfection Kit, Bulge-LoopTM mmu-U6 Primer Kit, Bulge-LoopTM mmu-miR-124-3p RT Primer, Bulge-LoopTM mmu-miR-124-3p primer and Bulge-Loop™ miR-Reverse Primer were purchased from Ribobio (Guangzhou, China). Adenovirus was purchased from OBiO Technology (Shanghai, China). Dual-Luciferase Reporter Assay System and pRT-TK vector were obtained from Promega (Madison, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), DMSO, LPS (055:B5) and other reagents were obtained from Sigma-Aldrich (St. Louis, USA).

## Cell culture

Murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, USA). Cells were cultured in DMEM supplemented with 10% (v/v) FBS and 0.5% (v/v) penicillin/streptomycin in a 5% CO₂ incubator at 37°C [14].

## Cell viability assay

Cell viability was determined by MTT assay as described previously [15]. Briefly, RAW 264.7 cells were seeded into a 96-well plate at a density of 5×10³ cells per well and incubated at 37 °C for 24 h. Then, cells were treated with different concentrations of FA for 48 h followed by incubating with 30 μl of MTT (5 mg/mL) per well for another 4 h at 37 °C. The supernatants
were removed and 100 μl of DMSO was added to dissolve the formazan crystals. The absorbance at 490 nm was measured using a microplate reader (Thermo Fisher Scientific).

**ELISA**

RAW 264.7 cells were cultured in a 24-well plate at a density of 3\times 10^4 cells per well and incubated at 37 °C. After 24 h, cells were treated with FA for 36 h, and then stimulated with LPS (100 ng/mL) for 9 h. The protein levels of IL-6 and TNF-α in cell supernatants and the level of IL-6 in bronchoalveolar lavage fluid (BALF) were determined by ELISA kit according to the manufacturer’s instructions as described previously [16].

**Immunofluorescence**

2 \times 10^5 RAW 264.7 cells were treated with FA (200 μM) for 36 h. Followed by co-incubating with LPS (100 ng/mL) for another 9 h, cells were washed with PBS (pH 7.2) trice, fixed with 4% (v/v) paraformaldehyde for 15 min and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 15 min. After blocked with 5% (w/v) BSA in PBS for 1 h, cells were incubated with STAT3 (1:800) or p-STAT3 (Tyr705) (1:100) antibody overnight. Then, cells were incubated with Alexa Fluor 488-conjugated anti-Rabbit IgG antibody (1:500) for 1 h in a blocking buffer and stained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min in darkness at room temperature (RT). Samples were washed with PBS trice and imaged under a confocal microscope (LSM800, Carl Zeiss, Oberkochen, Germany).

**Western blotting**
For the total protein extraction, RAW 264.7 cells were homogenized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1mM Na3VO4, 1 mM dithiothreitol, 1 mM phosphatase inhibitor) on ice for 15 min and vortexed for 45 s. Samples were centrifuged at 15 000 x g at 4 °C for 20 min and the supernatants were harvested and stored at −20 °C. (Lu et al., 2018). The protein concentration of protein extraction was quantified using a BCA kit. Proteins were separated by 10% SDS-PAGE and transferred onto Immunoblot polyvinylidene fluoride (PVDF) membranes. After blocked with 5% (w/v) skim milk dissolved in TBS-T for 1 h at RT. Immunoblotting was performed using appropriate primary antibodies: STAT3 (1:1000), p-STAT3 (Tyr705) (1:1000), β-actin (1:500). The membranes were incubated at 4 °C overnight, washed with TBS-T for three times and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1: 1000) for 1 h at RT. Membranes were washed with TBS-T for three times. Bands were detected using enhanced chamiluminescence (ECL) reagent and visualized by FluorChem E™ system (ProteinSimple, San Francisco, USA).

Quantitative real-time PCR

Total RNAs were extracted by using miRcute miRNA Kit according to the manufacturer’s instruction. The RNA quality and quantity were measured using a DS-11+ spectrophotometer (Denovix, Wilmington, USA). To analyze the expression of miR-124, RNAs were reversely transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser, and relative miRNA expression levels were determined by real-time quantitative PCR (qPCR) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus). The following primers were used for qPCR. β-actin: 5′-AGT GTG ACT GAC GTC gT-3′ (sense) and 5′-GCA GCT CAG TAA CAT GCC GC-3′ (antisense),
IL-6: 5'-TAG TCC TTC CTA CCC CAA TTT CC-3' (sense) and 5'-TTG GTC CTT AGC CAC TCC TTC-3' (antisense); STAT3: 5'-TGC TTG TCG GTT GGA GGT G-3' (sense) and 5'-GGG AAA GGA AGG CAG GTT GA-3' (antisense). Bulge-Loop™ mmu-U6 (for mature miR-124) and β-actin (for IL-6 and STAT3) were used as internal controls. All qPCR reactions were performed using LightCycler® 96 real-time PCR system (Roche, Basel, Switzerland), and the ΔΔCt method was used for the data analysis.

Dual Luciferase Reporter Assay

The 3'UTR sequence of STAT3 was subcloned into the pRT-TK vector. Renilla luciferase vector (pRL-SV40) was used to normalize the luciferase activity. Cells were infected by an adenovirus carrying the pRT-TK vector or Renilla luciferase vector. After cells were incubated with FA for 36 h, luciferase activity was determined using the Dual-Luciferase Reporter Assay System and a Wallac Victor3 1420 multilabel counter (PerkinElmer, Waltham, USA).

Transfection with miR-124 mimic or inhibitor

3×10⁵ RAW 264.7 cells were seeded in a 6-well plate. The miR-124 mimic (50 nM) or miR-124 inhibitor (200 nM) was transfected into cells with riboFECT™ CP Transfection Kit for 36 h according to the manufacturer’s instruction. qPCR was further conducted to examine the expression of miR-124.

Animals

All experimental protocols using 5 – 6 weeks old male BALB/c mice (18 – 22 g) were
purchased from the Center of Experimental Animals of Southern Medical University (Guangzhou, China), and performed in accordance with the ARRIVE guidelines and approved by the ethical committee for the experimental use of animals at Southern Medical University (no. L2019109) [17, 18]. The mice were housed in climate-controlled quarters (22 – 26 °C at 40 – 70 % humidity) with a 12 h light/12 h dark cycle. Mice were placed in cages (n = 4 mice per cage) and received food and water ad libitum. All animal experiments were conducted in the Laboratory Animal Centre of Southern Medical University.

Grouping and modeling

After acclimatized for one week, thirty-two mice were randomly allocated into four groups (n = 8 for each group). A co-worker blinded to the experimental protocol randomized animals into groups. Body weights of all animals were measured once a day during the experimental periods.

• Control group: These animals were received normal saline (200 μl, i.p.) for 7 days as the vehicle control. After that, animals were treated with the equivalent volume normal saline as used for the administration of FA groups and LPS group intratracheally on day 8.

• LPS group: This group was treated with LPS once on day 8 (3 mg/kg, intratracheal) [14].

• FA group: FA group was administered with FA (60 mg/kg, i.p.) once a day [19]. After 7 days, animals were treated with LPS (3 mg/kg, intratracheal) once.

• Antagomir (a miR-124 selective inhibitor) group: The animals were given Antagomir (10 μmol/kg, i.v.) on day 0, day 3 and day 6 and administered with FA (60 mg/kg, i.p.) daily. On day 8, animals were treated with LPS (3 mg/kg, intratracheal) once.

Before administration of LPS, mice were first anesthetized with 1.5% (w/v) pentobarbital
sodium solution. After instillation of LPS, mice were then allowed to recover until they awakened from anesthesia. Eight hours after the induction of injury, mice were anesthetized with 1.5% (w/v) pentobarbital sodium solution and sacrificed. A total of 1 mL of BALF samples and lung tissues were collected for further experiments.

**Histopathological observation of lung tissues**

The right lobes of lung tissues were isolated and flushed by cold saline, and immediately fixed in 4% (v/v) paraformaldehyde. After 24 h, the lung tissues were dehydrated with graded ethanol and embedded with paraffin. Lung specimens were cut into 5 μm thin sections. After deparaffinized and stained with hematoxylin and eosin (H & E, Yuanye Biotech, Shanghai, China), histological structures of each tissue section was observed under an IX 53 light microscope (Olympus, Tokyo, Japan). The histological scores were calculated based on the American Thoracic Society documents: An Official American Thoracic Society Workshop Report: Features and Measurements of Experimental Acute Lung Injury in Animals (2011).

**Lung wet/dry weight (W/D) ratio measurement**

The freshly harvested lung tissues were excised, blotted dry, and weighed by an electronic balance (Sartorius, Göttingen, Germany) to obtain the wet weight (W). Then, the samples were dried in a thermostatic dryer box (FUMA, Shanghai, China) at 60 °C. Until the weight was not changed, the dry weights (D) were recorded. To assess the degree of pulmonary edema, W/D ratio was determined by the following formula: W/D × 100 %.
After sacrifice of mice, the trachea was surgically exposed in the ventral neck area. The plastic cannula was inserted into the trachea. BALF was performed with two equals of 0.5 mL of cold NaCl solution (0.9%) instilled up to a total volume of 1.0 mL. BALF was centrifuged at 500 x g for 10 min at 4 °C. The protein concentration in the supernatant was measured by the BCA protein assay kit. The cell pellet was resuspended in 50 μl of NaCl solution and total BALF cells were counted double-blindly with a hemacytometer (Sigma-Aldrich).

Statistical analysis

Data were presented as mean ± SEM of at least five independent experiments. ANOVA was used to assess differences between multiple groups by SPSS 20.0 (IBM, Armonk, USA). Tukey’s test was used for multiple comparisons. \( P \leq 0.05 \) were considered as statistically significant.

Results

FA attenuates the release of IL-6 and TNF-α in LPS-stimulated RAW 264.7 macrophage cells

MTT assay was used to evaluate the cell viability of FA (Figure 1A) in RAW 264.7 macrophage cells. As shown in Figure 1B, FA under 200 μM had no obvious cytotoxicity in RAW 264.7 macrophage cells. Therefore, FA at the concentrations of 50 – 200 μM was selected in the subsequent experiments.

To determine the anti-inflammatory effect of FA on LPS-stimulated RAW 264.7 macrophage cells, we detected the release of pro-inflammatory cytokines IL-6 and TNF-α. Compared with
control group, LPS treatment stimulated the release of IL-6 and TNF-α which was significantly inhibited in the presence of FA. Moreover, FA (200 μM) showed the similar inhibitory activity in the production of IL-6 compared with dexamethasone (DEX) (100 μM) (Figure 1C, D). Collectively, these results showed that FA had a significant anti-inflammatory effects in the LPS-stimulated RAW 264.7 macrophage cells.

FA inhibits STAT3 in LPS-stimulated RAW 264.7 macrophage cells

STAT3 is the most crucial molecules in the IL-6 signaling pathway. After phosphorylation at Tyr705, STAT3 forms homo-dimers, which translocate into the nucleus, bind to the DNA and induce the production of IL-6 [20]. ELISA data showed that the inhibitory effect of FA on IL-6 was more obvious than TNF-α. Due to the close link of IL-6 and STAT3 in inflammation, we examined the expression and the transcriptional activity of STAT3. Western blotting and immunofluorescence results revealed that FA suppressed the expressions of p-STAT3 (Tyr705) and STAT3 (Figure 2A, B). Immunofluorescence results indicated that the expression of p-STAT3 and STAT3 were up-regulated both in the cytoplasm and nucleus, which was consistent with our previous study [21]. As a key transcription factor, STAT3 binds to consensus response elements in the promoters of target genes, thus inducing the transcription of specific genes, including IL-6 [22]. As shown in Figure 2C, luciferase reporter assay result revealed that FA inhibited the transcriptional activity of STAT3. Collectively, FA inhibited STAT3 in vitro.

FA attenuates inflammation by up-regulating miR-124 in LPS-stimulated RAW 264.7 macrophage cells
Previous research has reported that miRNAs are able to induce degradation of target mRNA or inhibiting its translation process to regulate inflammation. Therefore, we next identified which miRNA regulates STAT3 after the treatment of FA. The publicly available bioinformatics tools (TargetScan, miRTarBase and miRDB) showed that there are 9 miRNAs, including miR-124, miR-125a-5p, miR-130b, miR-874, miR-519d, miR-20b, miR-181a-5p, miR-17 and miR-20a, targeting to STAT3 (Figure 3A). Among these miRNAs, miR-124 has been reported to be a regulator of STAT3 and is associated with the release of IL-6 [23]. Therefore, we tested the expression of miR-124 in RAW 264.7 cells. We found that miR-124 was increased significantly after FA treatment, and peaked at 36 h (Figure 3B). Moreover, miR-124 was up-regulated in the combination of FA and LPS (Figure 3C). To further elucidate the role of miR-124 in anti-inflammatory effects of FA, We next utilized miR-124 mimic and inhibitor to up-regulate or down-regulate the expression of miR-124, respectively (Figure S1). As confirmed by qPCR, miR-124 mimic or inhibitor could up-regulate or down-regulate the miR-124 expression after treatment with LPS, respectively (Figure 4A). And miR-124 inhibitor attenuated the suppressive effects of FA on the mRNA levels of STAT3 and IL-6 (Figure 4B). The phosphorylation of STAT3 results in the dimerization and the nuclear translocation of STAT3 to induce the transcription of IL-6. Therefore, we investigated the protein expression level of p-STAT (Tyr705) and STAT3. Results showed that miR-124 inhibitor abolished the inhibitory effects of FA on p-STAT (Tyr705) and STAT3 (Figure 4C). Moreover, FA as well as miR-124 mimic attenuated the expression of STAT3 dimer in RAW 264.7 cells. Conversely, miR-124 inhibitor counteracted this effect of FA (Figure 4D). Consistently, the inhibition of miR-124 also decreased the suppressive effect of FA on the level of IL-6 (Figure 4E). These results revealed that FA inhibited
the activity of STAT3 and the production of IL-6 by up-regulating miR-124 in LPS-stimulated RAW 264.7 macrophage cells.

FA attenuates ALI via up-regulating miR-124

Inflammatory responses is pivotal in the progression of ALI. The main characteristics of ALI including non-cardiogenic pulmonary edema, hypoxemia and neutrophil infiltration are related to inflammation. MiRNAs, such as miR-100, miR-133a and miR-140 play an anti-inflammatory role in ALI [24]. Therefore, we detected that whether FA could prevent ALI through up-regulating miR-124. As shown in Figure 5A, B, FA or DEX reduced edema, hyperemia and inflammatory cells infiltration in LPS-induced ALI mice. FA or DEX also down-regulated the total protein (Figure 5C) and the total cells (Figure 5D) in BALF and lung W/D ratio (Figure 5E). For exploring the role of miR-124 in the anti-ALI effects of FA, miR-124 Antagomir (a miR-124 selective inhibitor) was used. According to our previous study, 10 μmol/kg Antagomir could significantly inhibited the expression of miR-124 in the lung tissues (Figure S2). Thus, Antagomir at the dose of 10 μmol/kg was selected for the subsequent experiments. As shown in Figure 5, the anti-ALI effects of FA were counteracted in the presence of miR-124 Antagomir.

We further detected the expression levels of miR-124 and related proteins in lung tissues. As shown in Figure 6A – C, FA up-regulated the expression of miR-124, and suppressed the phosphorylation of STAT3 at Tyr705 and the protein expression of STAT3. Moreover, FA inhibited the release of IL-6 in BALF from ALI mice, which was similar to DEX (Figure 6D). However, these effects of FA could be counteracted by inhibition of miR-124. Collectively, FA inhibited inflammatory responses in ALI mice through up-regulation of miR-124.
Discussion

Although treatment option and understanding of mechanism of ALI has made great progress, the mortality rate is still remains steady, which approaches 40% [25]. Forsythia suspensa, listed in Chinese Pharmacopoeia, is a traditional Chinese herb with the anti-inflammatory activity to treat lung infection [7]. It has been demonstrated that Phillyrin, one of the main chemical constituents of Forsythia suspensa, attenuated LPS-induced increase of TNF-α, IL-1β, IL-6 and inflammatory cells in BALF by suppressing the MAPK and NF-κB pathways in ALI [26]. Previous study merely showed that FA, the main natural product from Forsythia suspensa, protected mice from LPS-induced ALI [27]. However, the underlying molecular mechanisms are still unclear.

As we known, ALI is a life-threatening disease with severe inflammatory responses. Therefore, suppressing pulmonary inflammation is an important treatment in clinic. It has been documented that accumulation of inflammatory mediators in ALI, such IL-6 and TNF-α, could induced the “cytokine storm”, thereby resulting in immunosuppression and sepsis, multiple organ dysfunction syndrome and mortality [28]. Therefore, suppressing the releases of IL-6 and TNF-α is pivotal in ALI. As shown in our results, FA showed the similar inhibitory activity in the production of IL-6 compared with DEX in vitro and in vivo, indicating that FA exerts significant anti-inflammatory activity in LPS-induced inflammation. According to previous studies, DEX exhits proverbial side effects, such as decrease of body weight, atrophy of the adrenal and lymphoid organs [29]. Moreover, dexamethasone has teratogenic activity at the dose of 7.5 mg/kg in mice [30]. However, FA has no obvious toxicity in mice under the dose of 240 mg/kg [31], indicating the potential of FA to be the agent in the treatment of ALI.
STAT is a class of proteins, which regulate the expression of a large number of cytokines and
growth factors [32]. Current studies have found seven family members, including STAT1-4, STAT5a, STAT5b, and STAT6 [33]. As one of the seven family members, STAT3 stimulates the
transcription of many signaling factors to regulate the survival, growth and proliferation of cells [34]. STAT3 also plays a broad regulatory role in organisms, including inflammation [35].
Especially, STAT3 was considered to be a pivotal regulator impacting on response to acute inflammation [36]. STAT3 signaling can be activated by cytokines, interferons and growth factors, which leads to JAK family phosphorylation and then initiates phosphorylation of STAT3. After phosphorylation, STAT3 dimer is formed and then translocates to nucleus to conduct the
transcription of corresponding gene, including IL-6 [37]. Our study showed that FA has
significant anti-inflammatory effects as reflected by the decrease of TNF-α and IL-6. It should be pointed that the effect of FA on IL-6 is better than TNF-α. Therefore, we focused on the STAT3 pathway, which played a critical role on the regulation of IL-6. It has been conclusively demonstrated that STAT3 has two phosphorylation sites, Tyr705 and Ser727. Phosphorylation of Tyr705, the specific tyrosine residue in the transactivation domain (TAD), is required for the
activation of STAT3 [38, 39]. When phosphorylated, Tyr705 reinforces the protein-protein interaction of STAT3 to form homo-dimers. Tyr705 phosphorylation is triggered in ALI induced by LPS or IgG immune complexes. Inhibition of phosphorylation of STAT3 at Tyr705 results in reduce of edema, infiltration of inflammatory cells, alveolar collapse, and gene expression of IL-6 and IL-10 in lung tissues [40, 41]. However, the biological function of Ser727, the other phosphorylated site, is still controversial in STAT3 activation. Previous studies demonstrated that the phosphorylation of Ser727 inhibited STAT3 activity, whereas some research indicated that
Ser727 phosphorylation led to the maximum activation of STAT3 [42]. Only few studies reported that down-regulating Tyr727 phosphorylation of STAT3 contributed to the decrease of edema, neutrophils infiltration and alveolar septum thickness as well as the improvement of arterial blood oxygenation in ALI [43]. Our present data only indicated that FA could inhibit the Tyr705 phosphorylation of STAT3. Further investigation of the expression level and the role of Ser727 in the anti-inflammation effects of FA needs to be explored.

Besides the decrease of the phosphorylation of STAT3, FA also reduced the mRNA and protein expression level of STAT3 as well as the activity of STAT3 promoter. Thus, the underlying reasons were further explored. MiRNAs, with the functions of down-regulating the gene expression, binds with the untranslated regions of their targeted mRNAs, to inhibit the expression of the corresponding protein [44]. It has been previously demonstrated that numbers of miRNAs were involved in regulating STAT3 expression, including miR-124, miR-125, miR-21, miR-let-7, miR-148a, miR-98 and miR-17. MiR-let-7a overexpression suppresses the production of pro-inflammatory cytokine induced by α-synuclein in BV-2 microglia cell. It also found that miR-148a meliorates colitis by suppressing the expression of STAT3 and the pro-inflammatory cytokines in mice. Overexpression of miR-98 attenuated neuropathic pain development via targeting STAT3, thereby inhibiting the release of IL-6, IL-1β and TNF-α in chronic constriction injury rat models [45-47]. MiR-124, which was first discovered in mice in 2002, has a wide range of biological effects and is involved in cell proliferation, autophagy and neuronal differentiation [48, 49]. Moreover, miR-124 acts as an inhibitor in inflammatory diseases and immune disorders, such as chronic sinusitis, ulcerative colitis and rheumatoid arthritis [50-52]. Evidence revealed that STAT3 was the downstream target of miR-124. The up-regulation of miR-124 not only suppresses
LPS-induced IL-6 production by targeting STAT3 in macrophages, but also inhibits cell proliferation and induces apoptosis in non-small cell lung cancer through inhibiting STAT3 [23, 53]. However, miRNAs targeting STAT3 to attenuate ALI have not been reported. We found that FA up-regulated miR-124, and the blockage of miR-124 could abolish the inhibitory effects of FA on STAT3 (including the expression level of STAT3 and the formation of STAT3 dimer), and subsequently inhibiting the release of IL-6 both in vitro and in vivo, suggesting that FA exerted anti-inflammatory effects through up-regulating the expression of miR-124. It was noticeable that the inhibitory effect of FA on p-STAT3 (Tyr705) was greater than STAT3 and the inhibition of miR-124 attenuated the decrease of p-STAT3 (Tyr705) induced by FA. It has been validated that NADPH oxidase, tuberous sclerosis complex 2 and chemokine receptor CCR1 modulate the phosphorylation of STAT3, but have no effect on the expression of STAT3 in macrophage [54-55]. Thus, we speculated that the decrease of p-STAT3 (Tyr705) was partially caused by the increase of miR-124 and other modulators might be involved.

In conclusion, we first demonstrated that FA, the natural compound from Forsythia suspensa, possesses significant anti-inflammatory properties in vivo and in vitro through up-regulating miR-124 (Figure 7). By increasing the expression of miR-124, FA inhibits STAT3 and thereby suppresses the release of IL-6 to attenuate LPS-induced ALI. These findings provide a rationale for the application of FA as a potent agent in the treatment of ALI and indicate miR-124 may be a promising anti-ALI target.

Clinical perspectives

ALI is a global health issue with high mortality. Forsythia suspensa is commonly used in the
treatment of pneumonia in China for centuries with its anti-inflammatory activity. FA is the main bioactive ingredient of Forsythia suspense. Thereby, the objective of this work was to explore the anti-inflammatory effect and the underlying mechanism of FA in the LPS-induced ALI models. Using both cell and murine models of ALI, our results demonstrate that FA can alleviate the LPS-induced inflammation via up-regulating miR-124 expression. These results suggest that FA might partly contribute to the therapeutic effects of Forsythia suspense. FA may be a novel therapeutic drug to prevent the development of ALI.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author contributions

J.S.L. and L.Z.Y. designed the research study. Z.B.L. performed the research. Z.B.L., H.Y.Y., Y.Y.C., C.Y.H., H.L.Z. and H.H.C. performed the animal experiments. P.X. and D.Y.L. analyzed data. J.S.L. and Z.B.L. wrote the paper.
Abbreviations

ALI, acute lung injury; ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid;
BCA, bicinchoninic acid; DAPI, 4,6-diamidino-2-phenylindole; DEX, dexamethasone; DSS,
disuccinimidyl suberate substrate; ECL, enhanced chemiluminescence; FA, Forsythoside A; H &
E, hematoxylin and eosin; miRNAs, microRNAs; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2,
5-diphenyltetrazoliumbromide; NaCl, sodium chloride; Na$_3$VO$_4$, Sodium orthovanadate; Nrf2,
NF-E2-related factor 2; PVDF, polyvinylidenefluoride; RT, room temperature; Ser, Serine; TAD,
transactivation domain; TBS-T, tris-buffered saline containing Tween-20; Tyr, Tyrosine;
U-STAT3, Unphosphorylated STAT3.

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**Figure legend**

**Figure 1.** FA attenuates the production of IL-6 and TNF-α in LPS-stimulated RAW 264.7 cells. (A) The chemical structure of FA. (B) 50 – 200 μM of FA has no obvious cytotoxicity in RAW 264.7 macrophage cells. The cell viability of FA on macrophages was determined by MTT assay. Macrophages were incubated with FA for 24 h. (C – D) FA attenuates the production of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. After FA treated for 36 h, RAW 264.7 cells were cultured with LPS (100 ng/mL) for another 9 h. Quantitative analyses of TNF-α and IL-6 in supernatants of macrophages were measured by ELISA. Data are represented as the mean ± SEM (n = 5). *P < 0.05 by one-way ANOVA with Tukey’s test.

**Figure 2.** FA attenuates the activity of STAT3 in LPS-stimulated RAW 264.7 cells. (A – B) FA suppresses the expression of p-STAT3 (Tyr705) and STAT3. The RAW 264.7 cells were seeded and incubated with different concentrations of FA for 36 h and then stimulated with or without LPS (100 ng/mL) for 9 h. The expression of p-STAT3 (Tyr705) and STAT3 was detected by Western blotting (A) or immunofluorescence (B), respectively. (C) FA inhibits transcripational activity of STAT3. The RAW 264.7 cells were infected by adenovirus (2.00×10⁸ PFU/mL). 200 μM of FA was added to culture for another 36 h and then stimulated with LPS (100 ng/mL) for 9 h. The luciferase activity was measured by Dual-Luciferase® Reporter Assay System. Data are represented as the mean ± SEM (n = 5). *P < 0.05 by one-way ANOVA with Tukey’s test (A) or Student’s test (C).

**Figure 3.** FA increases the expression of miR-124 in RAW 264.7 cells. (A) The miRNAs
associated with STAT3 were predicted using publicly available bioinformatics tools (TargetScan, miRTarBase, and miRanda). (B–C) FA upregulates the level of miR-124. The RAW 264.7 cells were incubated with FA (200 μM) for different time points (B) or incubated with FA (200 μM) for 36 h and then stimulated with or without LPS (100 ng/mL) for 9 h (C). The expression of miR-124 was detected by qPCR. Data are represented as the mean ± SEM (n = 5). *P < 0.05 by one-way ANOVA with Tukey’s test.

**Figure 4.** FA suppresses the STAT3 signal pathway by up-regulating miR-124. (A) miR-124 mimic or inhibitor up-regulates or down-regulates the miR-124 expression in the presence of LPS, respectively. RAW 264.7 cells were transfected with miR-124 mimic (50 nM) or miR-124 inhibitor (200 nM) for 36 h. Then, cells were incubated with LPS (100 ng/mL) for 9 h. (B–E) After cells were transfected with miR-124 mimic or inhibitor for 36 h, FA (200 μM) was added to the FA group and the inhibitor group. After 36 h, LPS (100 ng/mL) was added to each group for 9 h except the control group. (B) miR-124 inhibitor antagonizes the inhibitory effects of FA on STAT3 mRNA and IL-6 mRNA. The expression of miR-124 was detected by qPCR. (C) miR-124 inhibitor counters the inhibitory effect of FA on the expression of STAT3 and p-STAT3 (Tyr705). The expression of STAT3 and p-STAT3 (Tyr705) was detected by Western blotting. (D) miR-124 inhibitor attenuates the inhibitory effect of FA on STAT3 dimer. Dimeric cross-linker disuccinimidyl suberate substrate (DSS) was used to cross-link the protein and Western blotting was used to detect the dimer content of STAT3. (E) miR-124 inhibitor abolishes the inhibitory effect of FA on the release of IL-6. The level of IL-6 was measured by ELISA. Data are represented as the mean ± SEM (n = 5). *P < 0.05 by one-way ANOVA with Tukey’s test.
Figure 5. FA inhibits LPS-induced ALI. (A – B) FA reduces edema, hyperemia and cells infiltration in LPS-induced ALI mice. The lung tissues were dehydrated and embedded in paraffin. After sectioning at 5 μm thickness, lung tissues were stained by H & E (40 ×) and histological score was estimated. (C – E) FA decreases the inflammation and edema induced by LPS. At 8 h after ALI, BALF was obtained to detect the total protein (C) and total cells count (D). Lungs were separated, and weighed to obtain the lung W/D ratio (E). Data are represented as the mean ± SEM (n = 8). *P < 0.05 by one-way ANOVA with Tukey’s test.

Figure 6. FA inhibits the STAT3 signal pathway by up-regulating miR-124 in ALI mice. After sacrifice, the lung tissues in ALI mice were harvested and the total proteins and RNA were extracted, respectively. (A) FA enhances the expression of miR-124 in ALI mice. The expression of miR-124 in lung tissues was measured by qPCR. (B – C) FA inhibits STAT3 activity in ALI mice. The protein expressions of STAT3 and p-STAT3 (Tyr705) were detected by Western blotting. (D) After mice were sacrificed, the BALF was obtained to measure the level of IL-6 by ELISA. Data are represented as the mean ± SEM (n = 8). *P < 0.05 by one-way ANOVA with Tukey’s test.

Figure 7. The speculated anti-inflammatory network of FA. ↓ indicates the inhibition. FA exerts anti-inflammatory effects through up-regulation of miR-124, and subsequently suppressing the STAT3 signal pathway to decrease the release of IL-6.
