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Research article

Forsythiaside A prevents zymosan A-induced cell migration in neutrophil-differentiated HL-60 cells via PD-1/PD-L1 pathway

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ABSTRACT

Neutrophils, which account for more than 80% of leukocyte, play an important role in resolution of inflammation. Immune checkpoint molecules could be potential biomarkers in immunosuppression. Forsythiaside A (FTA), a main constituent of *Forsythia suspensa* (Thunb.) Vahl, provides a very significant anti-inflammatory activity. Here we defined the immunological mechanisms of FTA by taking programmed cell death-1 (PD-1)/programmed cell death-Ligand 1 (PD-L1) pathway into consideration. FTA could inhibited cell migration in HL-60-derived neutrophils *in vitro*, and this action appeared to be mediated via PD-1/PD-L1 depended JNK and p38 MAPK pathways. *In vivo*, FTA prevented PD-L1$^+$ neutrophils infiltration and reduced the levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and interferon-gamma (IFN-γ) after zymosan A-induced peritonitis. PD-1/PD-L1 inhibitor could abolish the suppression of FTA. The expression of inflammatory cytokines and chemokines were positively correlated with PD-L1. Molecular docking showed that FTA could bind to PD-L1. Taken together, FTA might prevent neutrophils infiltration to exert inflammation resolution through PD-1/PD-L1 pathway.

1. Introduction

Inflammation is the reactions of immune system against infection or tissue damage and identified as a cause or consequence of many disease states such as trauma, sepsis, infections, and surgery [1,2]. Resolution of inflammation is a coordinated and active program to maintain tissue integrity and function [3]. Neutrophils constitute the largest circulating leukocyte population and the critical first line of cellular defense against infection. Their rapid recruitment from bone marrow to the infected/damaged sites is crucial for the elimination of microorganisms and the resolution of inflammation [4]. Activated neutrophils exert their potent action via degranulation responses, oxidative burst and NETosis [5,6]. Often, however, the continued and unlimited activation of neutrophils have deleterious effects on the host as a result of large amount and number of cytokines release, which further exacerbate inflammatory progression [7]. Therefore, it is imperative to restrain the neutrophils from entering circulation to affect the balance of the inflammatory response.

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Abbreviations

FTA	forsythiaside A
PD-1	programmed cell death-1
PD-L1	programmed cell death-Ligand 1
TNF-α	tumor necrosis factor alpha
IL-6	interleukin-6
MCP-1	monocyte chemoattractant protein-1
IFN-γ	interferon-gamma

Immune checkpoint inhibitors and immune costimulatory pathways maintain self-tolerance and prevent uncontrolled inflammation. Programmed death-1 (PD-1), membrane-bound type receptor, and its ligand PD-L1 (also termed B7–H1 or CD274) are major immune inhibitory checkpoint molecules that play critical roles in protection against excessive tissue damage induced by the immune response and autoimmunity, while its ligand PD-L2 (also termed B7-DC or CD273) is very limited. The PD-1/PD-L1 pathway has been found to be associate with septic morbidity, inflammatory cytokines and multiple organ damage [8–10]. In addition, PD-L1-expressing neutrophils increase at infectious site during inflammatory simulants, which supports PD-L1 as a mediator in inflammatory axis, even might be a candidate molecule to be analyzed to assess whether it correlated with immunosuppression, the risk of death and prognosis [11].

Forsythia suspensa Vahl. (*F. suspensa*) is a well-known traditional Chinese medicine native to China, Japan and Korea that has been widely reported as an important agent for erysipelas, ulcers and pyrexia [12]. Forsythiaside A (FTA) has been isolated from air-dried fruit of *Forsythia suspensa*, possessing anti-inflammatory, antioxidant and anti-bacterial activities [13,14]. Our recent studies showed that FTA (40 mg/kg) could markedly ameliorate mouse peritonitis induced by zymosan, and this was accompanied by reduced proportion of neutrophils and levels of inflammatory cytokines and chemokines in the peritoneal cavity [15]. However, the possible molecular mechanisms by which FTA produces these effects of inflammation-resolution have not been elucidated. According to these important roles of PD-1/PD-L1 pathway in the histological development of inflammation, we determine if PD-1/PD-L1 pathway contributes to the anti-inflammatory action of FTA.

2. Materials and methods

2.1. Reagents

Forsythiaside A (FTA, purity >98%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). BMS-202 was purchased from Meilunbio Biology Technology Co., Ltd. (Dalian, China). Zymosan A was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-mouse Gr-1-FITC (BM8, #123110), F4/80-PE (BM8, #123110), PD-L1-APC (10 F.9G2, #124311) and anti-human CD11b-APC (M1/70, #101212) mAbs were purchased from Biolegend (San Diego, CA, USA). Transwell chambers were purchase from Wuxi NEST Biotechnology Co., Ltd. (Wuxi, China). Wright-Giemsa Staining was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Real-time PCR kits were purchased from Shanghai Hifun Biotechnology Co. Ltd. (Shanghai, China). BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit was purchased from BD Co. (Franklin Lakes, NJ, USA). Antibodies against p38 (D13E1, #8690), p-p38 (D3F9, #4511), JNK (#9252), and *p*-JNK (81E11, #4668) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA).

2.2. Cell culture and differentiation

HL-60 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. To induce granulocytic differentiation, uninfected HL-60 cells were incubated with 1.25% DMSO in growth medium for 5 days. Cell morphology was assessed using Wright-Giemsa staining according to the manufacturer's protocol (Fig. S1). Cells were washed free from DMSO and cultured in fresh medium with serum for 24 h prior to use in experiments. All *in vitro* assays were performed after passage number 3 and before passage number 15 with cells growing at an exponential ratio.

2.3. Flow cytometry

For the expression of CD-11 b, neutrophi-like HL-60 cells were collected from cultures, washed twice with PBS, and then incubated with APC-conjugated anti-human CD-11 b or APC-conjugated IgG1 isotype control antibody for 30 min on ice. Cells collected from peritoneal cavity were washed and incubated with anti-mouse mAbs Gr-1-FITC, F4/80-PE and PD-L1-APC (all from biolegend) for 30 min at 4 °C as described previously [15]. Data were acquired on CytoFLEX S (Beckman Coulter) and analyzed with Flowjo software (TreeStar, Ashland, OR, USA).

2.4. Cell viability assay

The viability of neutrophil-like HL-60 cells was evaluated by CCK-8 assay. Cells were seeded in flat bottomed 96-well plates at an initial density of 1×10^4 cells/well with 200 μl medium and treated with serial dilutions of FTA (1.25, 5, 10, 20, 40 and 80 μM) for 24 h. Serum free medium was set as negative control. 20 μL of CCK-8 reagents was added to each well 4 h before the end of incubation, and the optical absorbance was detected at wavelength of 450 nm using a Microplate Reader (VersaMAx, Molecular Devices).

2.5. Cell migration assay

The migration capability of the neutrophil-like HL-60 cells was examined in transwell chambers (Corning, Costar, USA). Differentiated HL-60 neutrophil-like cells were stimulated with zymosan (10 μg/mL) for 30 min. After pretreated with or without BMS-202 (a PD-1/PD-L1 inhibitor) (0.1 μM), GDC-0941 (a PI3k inhibitor) (1 μM), SP600125 (a JNk inhibitor) (10 μM) or SB203580 (a p38 MAPK inhibitor) (10 μM), cells were treated with FTA (2.5, 5, 10 μM) or BMS-202 (2 μM). Then, the cells (1×10^6 cells/well) were placed in the upper chambers, and RPMI 1640 medium was added into the lower chambers. After 4-h incubation at 37 °C, cells attached to the lower chambers were collected and counted by cell counting chambers.

2.6. Western blotting

The total cell lysates from HL-60 neutrophil-like cells were prepared by suspending cells in NP40 buffer (Beyotime, Shanghai, China). Protein concentrations were determined by Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher, MA, USA). Equal amounts (30 μg) of extracted proteins were separated on 10% SDS-PAGE gels, transferred electrophoretically onto PVDF membranes, and blocked with 5% non-fat milk for 1 h. The membranes were probed with specific primary antibodies overnight at 4 °C and then incubated with anti-rabbit or anti-mouse HRP antibody for 1 h at room temperature. Primary antibodies and dilution were as follows: total JNK (1:1000), phosphor-JNK (1:2000), total p38 (1:1000) and phophor-p38 (1:1000). GAPDH (1:1000) was set as a loading control. The immunoreactive bands were visualized using a chemiluminescence imaging system (ChemiDoc™ XRS + System, Bio-Rad Laboratories, Inc.).

2.7. Animals

Male C57BL/6 J mice aged 6–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and housed in the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine accredited by the Institutional Animal Care and Use Committee in Institut Pasteur of Shanghai (No. SZY 201804005) under standardized conditions (12 h light-dark cycle and 22 ± 2 °C) with food and water ad libitum.

The acute peritonitis model was established by intraperitoneal injection of 1 mg zymosan A in 0.5 mL sterile 0.9% phosphate buffered saline. Mice were assigned randomly into six experimental groups (6 mice per group): normal group, model group, FTA (40 mg/kg, i. p.), BMS-202 (25 μg/per mouse, i. p.), FTA + BMS-202 (25 μg/per mouse, i. p.) and BMS-202 (200 μg/per mouse, i. p.). FTA (40 mg/kg) or BMS-202 (200 μg) was injected 30 min after zymosan treatment. Normal mice received only sterile 0.9% phosphate buffered saline at the same time points. The co-administration of BMS-202 (25 μg, i. p.) was conducted 30 min before FTA treatment. The peritoneal cavity was laved 4 h after zymosan A injection. Peritoneal cells and the supernatant were collected for flow staining and cytokines analysis.

2.8. Cytokine and chemokine assays

Analysis approach was performed by allowing quantification of TNF-α, IL-6, MCP-1 and IFN-γ using BD CBA Mouse Inflammation Kit on a FACSCalibur cytometer equipped with CellQuestPro and CBA software (BD Becton–Dickinson), according to the manufacturer's instructions.

2.9. Quantitative real-time (QRT) PCR assay

Total RNA was isolated from cells using *EZ*Bioscience™ Tissue RNA Purification Kit PLUS, following the manufacturer's instructions. Real-time PCR assay was conducted on 7500 Real Time PCR System using *EZ*BioscienceTM qPCR SYBR Green Master Mix. The specific primers for amplifying genes were: TNF-α, Forward: 5′-TCTTCTCATTCCTGCTTGTGG-3′, Reverse: 5′-GGTCTGGGCCATAGAACTGA-3'; IL-6, Forward: 5′-GGAGCCCACCAAGAACGATAG-3′, Reverse: 5′-GTGAAGTAGGGAAGGCCGTG-3'; IFN-γ, Forward: 5′-TGGCTGAACTGTCGCCAGCA-3′, Reverse: 5′-TGGCTGCCTAGTTGGCCCCT-3'; MCP-1, Forward: 5′-GGCTCAGCCAGATGCAGTTAA-3′, Reverse: 5′-CCTACTCATTGGGATCATCTTGCT-3'; PD-1, Forward: 5′-TTCACCTGCAGCTTGTCCAA-3′, Reverse: 5′-TGGGCAGCTGTATGATCTGG-3'; PD-L1, Forward: 5′-AAAGTCAATGCCCCATACCG-3′, Reverse: 5′-TTCTCTTCCCACTCACGGGT-3'; GAPDH, Forward: 5′-GGTGAAGGTCGGTGTGAACG-3′, Reverse: 5′-CTCGCTCCTGGAAGATGGTG-3'; human MCP-1, Forward: 5′-TTGATGTTTTAAGTTTATCTTTCATGG-3′, Reverse: 5′-CAGGGGTAGAACTGTGGTTCA-3'; human GAPDH, Forward: 5′-TGACTTCAACAGCGACACCCA-3′, Reverse: 5′-CACCCTGTTGCTGTAGCCAAA-3'. The expressions of genes were normalized to GAPDH, and the results were evaluated by using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

2.10. Molecular docking

The three-dimensional crystal structure of PD-L1 was downloaded from the Protein Data Bank code 5 × 8M. The protein was prepared using the protein preparation wizard of LigPrep module of Schrödinger 2021–3. Using molecular docking technique, the best model was obtained by glide score, which was generated by the best fit of the ligand and the receptor. The final model was subjected to a binding site analysis and docking study.

2.11. Statistical analysis

The values are represented as mean ± SEM. Statistical analysis was performed with GraphPad Prism 5.0 software (San Diego, CA, USA). Significant differences between groups were determined using one-way analysis of variance (ANOVA) and *post hoc* Tukey's test. $P < 0.05$ was considered to show a statistically significant difference.

3. Results

3.1. Effect of FTA on zymosan-induced migration of neutrophi-like HL-60 cells

DMSO (1.25%)-treated HL-60 cells were cultured to differentiate into neutrophils for *in vitro* studies. As an indicator of neutrophil-like differentiation, undifferentiated HL-60 cells presented essentially no CD11b, while DMSO treated cells had been increased to 93.3% (Fig. 1A). To rule out the probability that the impact of FTA was due to cytotoxicity, we assessed cytotoxicity of FTA against differentiated HL-60 neutrophil-like cells. The data showed that FTA (1.25–40 μM) did not affect the viability of neutrophi-like HL-60 cells, while FTA (80 μM) obviously inhibited the viability (Fig. 1B). We then explore the suppressive impact of FTA on neutrophi-like HL-60 cell migration *in vitro*. Results indicated that FTA (10 μM) significantly inhibited zymosan-induced HL-60 neutrophil-like cell migration in transwell migration assay and concentration-dependently inhibited the mRNA expression of MCP-1, which was a key chemokine that regulated cell migration and infiltration. (Fig. 1C and D). Notably, FTA (5, 10 μM) almost completely abrogated the elevated MCP-1 expression induced by zymosan on differentiated HL-60 neutrophil-like cells.

3.2. PD-1/PD-L1 inhibitor reversed the anti-migration effect of FTA on neutrophi-like HL-60 cells through JNK and p38 MAPK signaling pathways

To illustrate the part that PD-1/PD-L1 pathway played in the immunosuppressive effect of FTA on neutrophils migration, BMS-202, which induced PD-L1 protein dimerization to inhibit PD-1/PD-L1 interaction, was used as a PD-1/PD-L1 pathway inhibitor at a low concentration. Meanwhile, BMS-202 could effectively inhibit proliferation, migration, and boost immunosuppressive with no significant cytotoxicity [16], was chosen as a positive control for *anti*-PD-1/PD-L1 therapy. BMS-202 (0.1 μM) itseslf did not cell

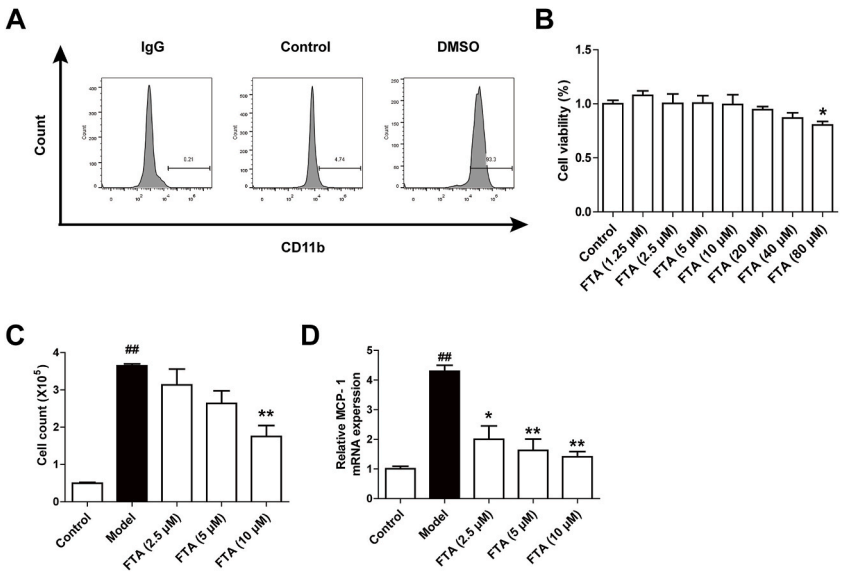

Fig. 1. Influence of PD-1/PD-L1 pathway on the inhibition of zymosan-induced neutrophils migration by FTA. (A) DMSO treated HL-60 differentiation. CD11b expressions on intact HL-60 cells and DMSO-differentiated cells were determined by flow cytometry. (B) Cell viability of neutrophil-like HL-60 cells was assessed by CCK-8 assay. (C) The effect of FTA on zymosan-induced neutrophil-like HL-60 cells migration was examined by transwell chamber assay. (D) The effect of FTA on MCP-1 mRNA level in neutrophil-like HL-60 cells was evaluated by QPCR assay. Data were expressed as means ± SEM (n = 3). $^{\#\#}P < 0.01$ *vs*. Normal; $^{*}P < 0.05$ *vs*. Model; $^{**}P < 0.01$ *vs*. Model.

migration, but it almost completely prevented the anti-migration properties of FTA. BMS (2 μM) markedly inhibited zymosan-induced cell migration (Fig. 2A and B). To examine the signaling pathways involved in the inhibition by FTA on cell migration, GDC-0941, SP600125, SB203580 were co-added with FTA. SP600125 and SB203580 markedly diminished the inhibition of MCP-1 mRNA caused by FTA in HL-60 neutrophil-like cells, while GDC-0941 did not influence the part of FTA on MCP-1 levels (Fig. 2C). BMS-202 (0.1 μM) diminished the phosphorylation of p38 and JNK inhibited by FTA (Fig. 2D). These results highlighted the fact that PD-1/PD-L1 pathway was responsible for the inhibition effect on neutrophi-like HL-60 cell migration of FTA.

3.3. PD-1/PD-L1 pathway was vital for the inflammation-resolution of FTA

To illustrate the part that PD-1/PD-L1 pathway played within the inflammation-resolution of FTA, *in vivo* studies were performed in zymosan-induced peritonitis in mice, which is a self-resolving acute inflammation model. As shown in Fig. 3A, zymosan treatment led to a significant rise in neutrophils in peritoneal cavity at 4 h during the resolution phase. BMS-202 (25 μg) had no significantly influence on the number neutrophils in mice. FTA (40 mg/kg) and BMS-202 (200 μg) markedly reduced neutrophils numbers when compared to the model group. The mice co-administrated with FTA and BMS-202 (25 μg) exhibited increased neutrophils numbers. To ascertain tissue-specific PD-1 and PD-L1 expression in the inflammation-resolution of FTA, we first observed the mRNA expressions of these molecules in peritoneal cavity cells in response to zymosan-induced peritonitis. Data showed that FTA treatment markedly decreased the mRNA expression of PD-L1 compared to the expression of PD-1, which was partially diminished by BMS-202 (25 μg) (Fig. 3B). We next inspected PD-L1 expression on peritoneal neutrophils. The level of PD-L1 on neutrophils was enhanced during peritonitis. FTA (40 mg/kg) markedly decreased PD-L1 expression compared to model group, which was almost completely abolished by co-administration of BMS-202 (25 μg) (Fig. 3C). These results implied that PD-L1 might be pivotal for FTA to suppress neutrophil infiltration.

3.4. FTA inhibited cytokines levels in the peritoneal cavity through PD-L1

Inflammation often associates with dysfunctional development characterized by certain cytokines/chemokines. The role of PD-L1 in the anti-inflammatory effects of FTA on cytokines expressions was assessed. As shown in Fig. 4A, FTA (40 mg/kg) obviously reduced the expressions of TNF-α, IL-6, MCP-1 and IFN-γ in peritoneal cavity, BMS-202 (25 μg) completely reversed the inhibitory impact of FTA on pro-inflammatory cytokines and chemokines levels. Of note, BMS-202 (25 μg) did not influence the part of FTA in reducing the level of IFN-γ, which is an innate immunity-related cytokine. Similar results were obtained in mRNA expressions of cytokines (Fig. 4B).

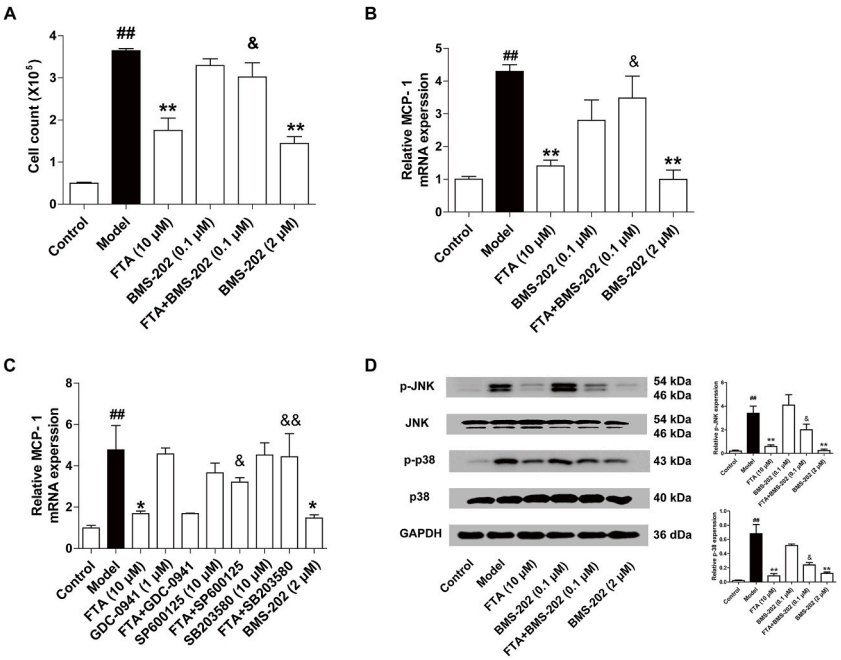

Fig. 2. Involvement of PD-L/PD-L1 and relative signaling pathways in anti-migration effect of FTA in neutrophil-like HL-60 cells. (A) Migration of neutrophil-like HL-60 cells was determined by transwell chamber assay. (B, C) The relative MCP-1 mRNA expression in neutrophil-like HL-60 cells was determined by QPCR assay. (D) The protein levels of p38 and JNK were detected by Western blot. Original images were shown in Fig. S2. Data were expressed as means ± SEM (n = 3). $^{\#\#}P < 0.01$ *vs*. Normal; $^{*}P < 0.05$ *vs*. Model; $^{**}P < 0.01$ *vs*. Model; $^{\&}P < 0.05$ *vs*. FTA; $^{\&\&}P < 0.01$ *vs*. FTA.

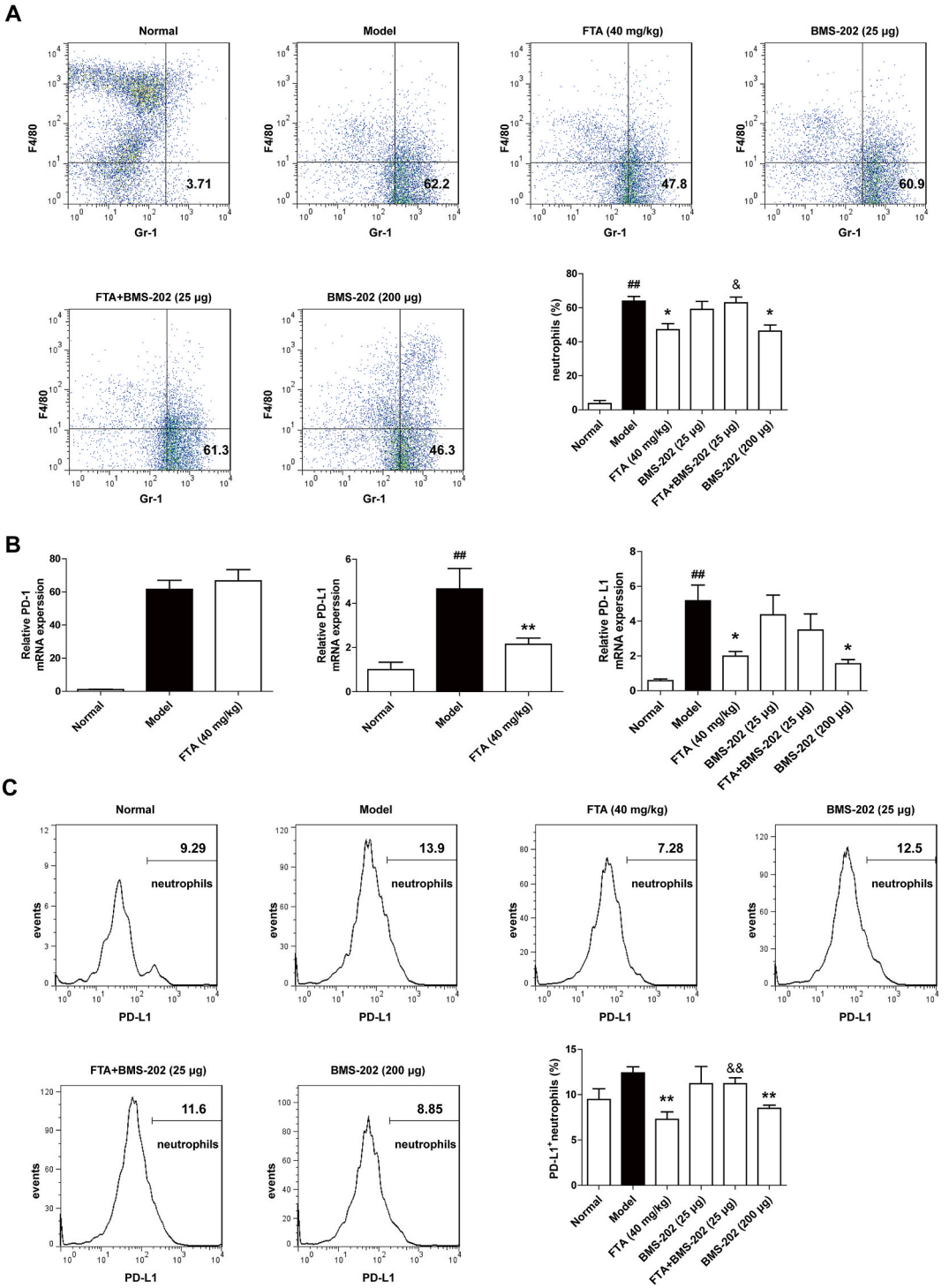

Fig. 3. Influence of PD-1/PD-L1 inhibitor on the anti-inflammatory effect of FTA induced by zymosan in mice. Cells and supernatants were centrifuged and collected from peritoneal cavity of zymosan injected mice. (A) The extents of neutrophils were determined by flow cytometry. (B) Levels of PD-1 and PD-L1 were quantified by QRT-PCR. (C) The extents of PD-L1^{+}neutrophils were estimated by flow cytometry. Data were expressed as means ± SEM (n = 6). $^{\#\#}P < 0.01$ *vs.* Normal; $^{*}P < 0.05$ *vs.* Model; $^{**}P < 0.01$ *vs.* Model; $^{\&}P < 0.05$ *vs.* FTA; $^{\&\&}P < 0.01$ *vs.* FTA.

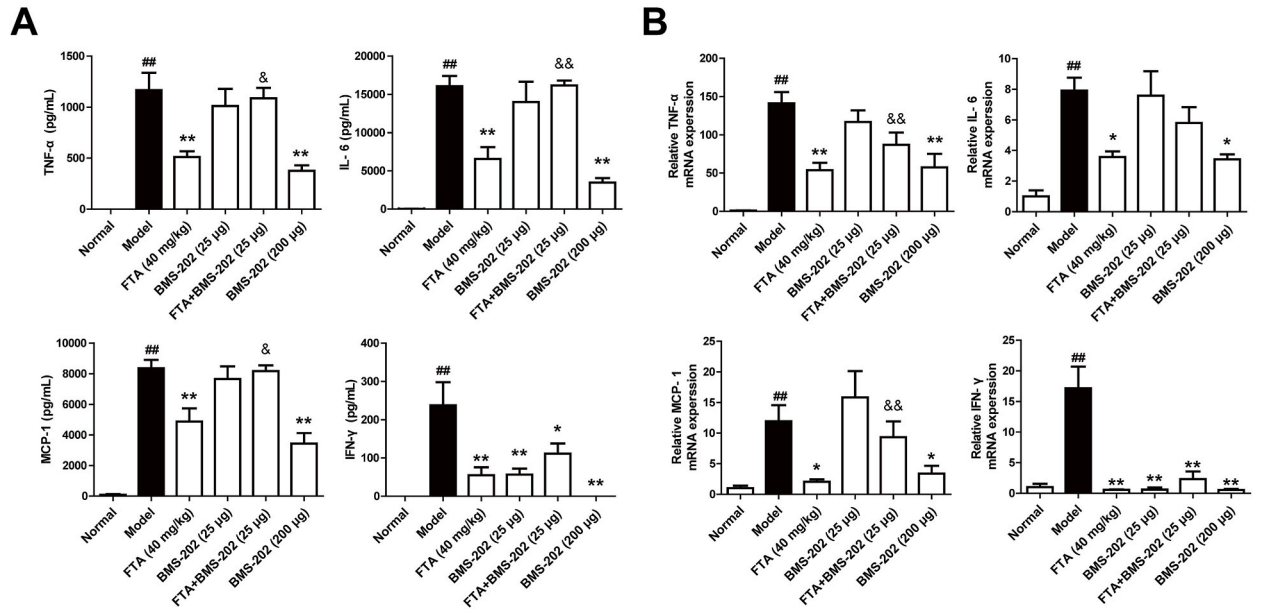

Fig. 4. Influence of PD-1/PD-L1 inhibitor on the anti-cytokine effect of FTA induced by zymosan in mice. (A) The cytokines expressions were measured by BD CBA Mouse Inflammation Kit. (B) The mRNA levels of cytokines were quantified by QRT-PCR assay. Data were expressed as means ± SEM (n = 6). $^{\#\#}P < 0.01$ *vs*. Normal; $^{*}P < 0.05$ *vs*. Model; $^{**}P < 0.01$ *vs*. Model; $^{\&}P < 0.05$ *vs*. FTA; $^{\&\&}P < 0.01$ *vs*. FTA.

3.5. *The mRNA levels of cytokines in the peritoneal cavity of FTA-treated mice positively correlated with PD-L1*

To explore whehter the expressions of cytokines in peritoneal cavity were associated with PD-L1, a spearman bivariate correlation analysis was employed. As Fig. 5A–D showed, the peritoneal cavity levels of TNF-α, IL-6 and MCP-1, but not IFN-γ, in FTA-treated mice were highly positively correlated with PD-L1, suggesting that PD-L1 might be the key mediator responsible for anti-inflammatory effect of FTA.

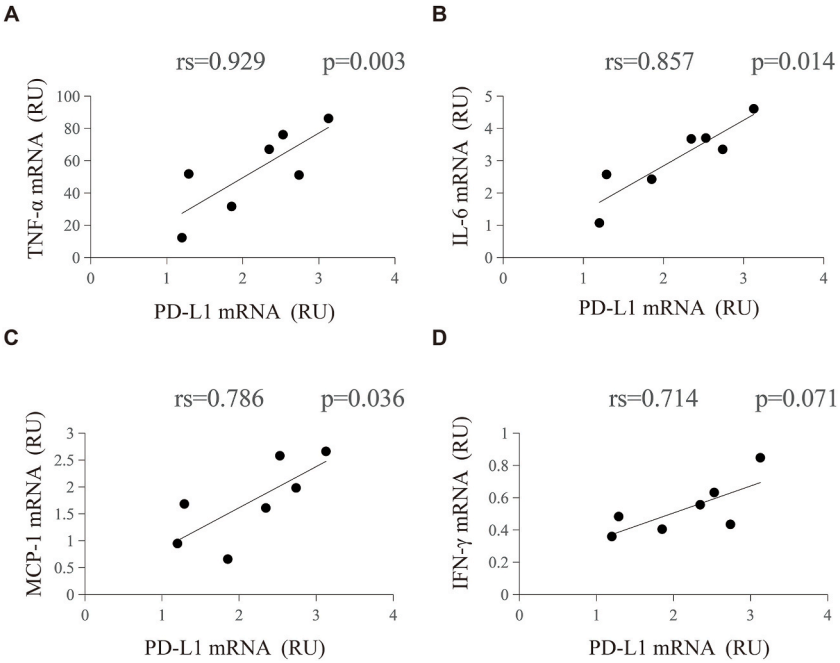

Fig. 5. Influence of PD-1/PD-L1 inhibitor on the anti-cytokines effects of FTA induced by zymosan in mice. Association of PD-L1 mRNA expression in the peritoneal cavity. Spearman's rank correlation coefficient of PD-L1 with TNF-α (A), IL-6 (B), MCP-1 (C) and IFN-γ (D) mRNA in FTA-treated peritonitis mice. RU = relative unit. *P*-values less than 0.05 were considered to show significant differences.

3.6. Molecular docking of FTA binding to PD-L1

To further recognize whether FTA can specifically interact with PD-L1, a molecular simulation of FTA-PD-L1 complex using LigPrep module of Maestro by Schrödinger was performed. As shown in Fig. 6, the key residues around FTA included Glu71, Glu72, Gln77, Lys89, Arg86, Gln83, Asp90. This result implied that FTA might directly bind to PD-L1.

4. Discussion

PD-1/PD-L1 pathway has been demonstrated to be widely expressed in many types of cancer and immune cells and is a vital part in a large spectrum of immune responses [17–19]. PD-L1 exacerbates disease severity in inflammatory diseases such as experimental autoimmune encephalomyelitis, pancreatitis and multiple sclerosis, whereas PD-L1 antagonists decrease inflammation and improve disease symptoms [20,21]. According to the findings we previously reported, FTA, extracted from *Forsythia suspensa*, can markedly alleviate zymosan-induced acute peritonitis [15]. The objective of this study was to address the immune system underlying mechanisms in relation to PD-1/PD-L1 pathway and subsequently mediated the process in inflammation-resolution. The results showed that FTA functions by binding to and inhibiting PD-L1, inhibiting MCP-1 levels and relative signaling pathways including JNK and p38 MAPK.

Neutrophils are the principal effectors as the first step of host defense against injury or infection [22]. They are recruited to inflammatory sites, where they generate potent destructive enzymes and cytokines and phagocytose potentially injurious stimuli [23,24]. PD-1/PD-L1 pathway participates in inflammatory response and maintains immune homeostasis [8]. Blocking PD-1/PD-L1 axis has been used in viral infectious diseases, such as HIV and tuberculosis [25,26]. Our findings demonstrated that PD-1/PD-L1 were up-regulated in the peritoneal cavity in experimental peritonitis mice, which is consistent with prior researches that PD-1/PD-L1 pathway was activated in the early phase of sepsis-induced immune suppression [27,28]. In consistent with the previous report that PD-L1 expression correlated negatively with tissue destruction and neutrophil lung infiltration in inflammatory diseases such as chronic obstructive lung disease and sepsis [29,30], our results showed that high concentrations of BMS-202 (PD-1/PD-L1 inhibitor) could reduce inflammation. On the basis of our prior studies that FTA had pro-resolving activity through inhibiting zymosan-induced neutrephils infiltration and inflammatory cytokines [6], the inhibition of neutrophils influx might be depend on PD-1/PD-L1 pathway.

Chemokines contribute to the pathogenesis of several diseases including rheumatoid arthritis, asthma, and sepsis [31,32]. MCP-1, a part of *C*–C chemokine family, controls the recruitment of neutrophils and Tregs [33]. As an immunosuppressive molecule in the development of peritonitis, we attempted to detect MCP-1 in mouse peritoneal cavity and neutrophil-like cells. Our results showed that FTA could inhibit PD-L1 and MCP-1 *in vivo* and *in vitro*. These findings suggested that MCP-1 down-regulation caused by PD-L1 inhibition could be a critical step in the PD-1/PD-L1 pathway function, which was the same with other groups [34,35]. According these evidences, we concluded that FTA mediated immunosuppression through inhibiting PD-L1 and consequently down-regulating the expression of MCP-1 in neutrophils. However, further experiments are needed to thoroughly clarify the potential of FTA to bind PD-L1.

IFN-γ is a key cytokine in innate immune responses leading to many cellular immunity including cytotoxicity, proliferation, apoptosis [36]. In addition, it has been promoted as a possible treatment for sepsis-induced immune suppression [37]. Recently, IFN-γ was reported to induce PD-L1 gene expression and PD-L1^{+} neutrophils production [38]. *In vitro* stimulation with IFN-γ resulted in PD-L1 production in neutrophils, which reduced the proliferation of T-lymphocyte [39]. However, a relationship exists between innate and adaptive immunity in the resolution of acute inflammation [40]. Furthermore, the regulation of PD-L1 expression on immune cells is partially regulated by IFN-γ [41]. This may provide an explanation as to why FTA inhibited the expression of IFN-γ without interfering PD-1/PD-L1 pathway.

At present, the molecular mechanism of PD-L1 expression in neutrophils induced by zymosan is not clear in immunosuppressive process. Researchers have found that the JNK, p38 MAPK, and PI3k/Akt pathways are involved in the signal transduction of MCP-1 in neutrophils [42,43]. According to our early findings, inhibitors of JNK and p38 MAPK might effectively counteract the effect of FTA on MCP-1 production.

According to our current findings, FTA decreased MCP-1 production in neutrophi-like HL-60 cells through inhibiting the PD-L1 and JNK and p38 MAPK pathways.

Because the precise process of MCP-1 synthesis is still unknown, more research is needed to determine the association between JNK and p38 MAPK pathways inhibition and MCP-1 expression especially in neutrophils.

5. Conclusion

In summary, FTA suppresses neutrophils infiltration through inhibiting PD-L1 and subsequently inhibiting the JNK and p38 MAPK signaling pathways. These findings demonstrate that targeting the PD-1/PD-L1 pathway could be a promising therapy for patients with peritonitis and with other acute inflammation characterized by neutrophils infiltration. The present study also provides a paradigm for the mechanistic studies for the inflammation-resolution effect of FTA.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

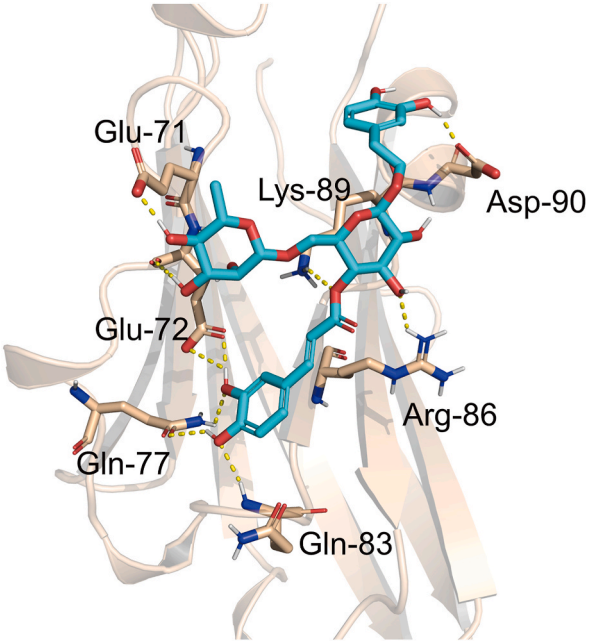

Fig. 6. Molecular docking study of FTA binding to PD-L1. The theoretical possibility of FTA binding to PD-L1 was performed using LigPrep module of Maestro.

Author contribution statement

Xinyu Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Aiyun Li: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Yue Xu: Performed the experiments; Analyzed and interpreted the data.

Jinshuai Lan; Yun Liu; Ling Li: Performed the experiments.

Ping Kang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tong Zhang: Conceived and designed the experiments.

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Data availability statement

Data included in article/supp. Material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13490.

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