Forsythoside A exerts an anti-endotoxin effect by blocking the LPS/TLR4 signaling pathway and inhibiting Tregs *in vitro*

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Abstract. Endotoxins, also referred to as lipopolysaccharides (LPS), are powerful immunostimulators involved in a number of severe diseases. Forsythoside A (FTA), a monomer of phenethyl alcohol glycosides extracted from *Forsythia suspensa*, has been shown to possess anti-bacterial and immunomodulatory properties. However, it is currently not known whether FTA can counter the adverse effects of endotoxins. We investigated the effect of FTA on LPS-stimulated RAW264.7 cells and primary lymphocytes to determine its molecular mechanism of action. RAW264.7 cells and primary lymphocytes were incubated with or without LPS (100 ng/ml) in the presence or absence of FTA or polymyxin B. We found that FTA increased the viability of LPS-treated RAW264.7 cells and primary lymphocytes suggesting that FTA effectively counters the adverse effects of endotoxins. FTA decreased the percentage of regulatory T cells (Tregs) and inhibited the TLR4/MyD88/NF-κB signaling pathway, downregulating Foxp3, IL-10 and TGF-β1, molecules involved in the immunosuppressive function of Tregs. These findings elucidate the molecular mechanism underlying the anti-endotoxin effects of FTA and suggest its use as a new treatment for LPS-induced diseases.

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Abbreviations: ALI, acute lung injury; DMEM, Dulbecco's modified Eagle's medium; FTA, forsythoside A; FBS, fetal bovine serum; IL, interleukin; LPS, lipopolysaccharides; PAMP, pathogen-associated molecular patterns; NF-κB, nuclear factor-κB; PAMP, pathogen-associated molecular patterns; PMB, polymyxin B; TCR, T-cell receptor; TLR, Toll-like receptors; TNF, tumor necrosis factor; Tregs, regulatory T cells

Key words: forsythoside A, anti-endotoxin, lipopolysaccharides, Toll-like receptor 4, regulatory T cells

Introduction

Endotoxins, also referred to as lipopolysaccharides (LPS), are considered as the most powerful stimulators of the immune system and measurement of their levels is considered to be a useful specific indicator of infection by Gram-negative bacteria in diverse eukaryotes, ranging from insects to humans (1). LPS are present in many liquids or many biomaterials, even if the material is sterile (2), and can elicit a biological effect even at extremely dilute concentrations (3). High levels of endotoxins in the blood can cause various disease status, including systemic inflammatory response syndrome, sepsis, severe shock, multiple organ dysfunction syndrome, multiple organ failure and even death (4). Despite the recognized potential for causing harm, there is currently no effective method available for treating LPS-induced diseases.

LPS and other microbial products are recognized by the innate immune system of the body through the action of Toll-like receptors (TLRs), leading to activation of the downstream signal transduction pathways and stimulation of a range of immune responses (5,6). TLR4 is the first TLR discovered in humans (7,8) and is capable of identifying bacterial LPS. LPS binding to TLR4 triggers myeloid differentiation through primary response gene-88 (MyD88)-independent pathways (9), leading to subsequent activation of nuclear transcription factor nuclear factor-κB (NF-κB). Activated NF-κB translocates to the nucleus and mediates the transcription of a number of genes (10). Studies have shown that NF-κB can effectively induce the expression of genes encoding inflammatory mediators and stimulate their release from cells (11). Therefore, treatments aimed at inhibiting the TLR4/MyD88/NF-κB signaling pathway may have potential therapeutic advantages over currently available approaches for managing the effects of LPS. However, the inhibition of the TLR4/MyD88/NF-κB

signaling pathway may also damage the anti-microbial immunity of the host.

Upon encountering pathogens and environmental insults, recruitment and activation of effector T and B cells and, importantly, regulatory T cells (Tregs) contribute to the maintenance of immune homeostasis, prevention of autoimmunity and moderation of the inflammatory response (12). Tregs are a functionally mature subpopulation of T cells, characterized as $CD4^+/CD25^+/Foxp3^+$. Tregs are generated in the thymus or by differentiation from peripheral $CD4^+CD25^-$ native T cells (13). Tregs play key roles in the maintenance of immunologic self-tolerance and negative control of a number of physiological and pathological immune responses, with growing recognition of the clinical importance of Tregs. Since TLR4 is expressed on Tregs, LPS may affect their function through stimulation of TLR4. Foxp3 protein is considered to be the most reliable molecular marker of mature Tregs and is involved in the development and function of Tregs. Several mechanisms of Treg-mediated suppression of the immune response have been proposed, including secretion of immunosuppressive cytokines, cell-contact-dependent suppression and functional modification or killing of antigen-presenting cells (12,14), among others. Tregs are unresponsive to T cell receptor (TCR) stimulation, express transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), inhibit normal T cell proliferation, and suppress $CD4^+$ expansion *in vivo*.

Many researchers are engaged in identifying novel active compounds from plants for the treatment of human diseases. Qinghaosu (artemisinin) used as an antimalarial drug is well known (15). Compound Danshen dropping pill has been widely used for cardiovascular disease in China and some Asia countries (16). Moreover, numerous traditional medicines have anti-endotoxin effects such as andrographolide, resveratrol, praeruptorin and osthole (17-20). Forsythoside A (FTA) (Fig. 1) is a pharmacologically active monomer of benzene glycoside extract from the *Forsythia suspensa* plant. It was reported to possess anti-bacterial, anti-viral, anti-oxidant, and immunomodulatory properties (21). Recent studies have shown that forsythoside can significantly enhance macrophage phagocytosis and reduce tumor necrosis factor-α (TNF-α) secretion in LPS-stimulated RAW264.7 cells, thereby suggesting that it can elicit various anti-LPS effects (22). However, the precise anti-endotoxin effects of FTA have not been elucidated to date.

In the present study, we investigated the ability of FTA to suppress the effects of LPS *in vitro*. Furthermore, we evaluated whether the effects involve the LPS/TLR4/MyD88/NF-κB signaling pathway and inhibition of Tregs.

Materials and methods

Reagents. Bacterial LPS (*Escherichia coli*: 055:B5, used at 100 ng/ml) and polymyxin B (PMB, used at 10 μg/ml) were obtained from Sigma (St. Louis, MO, USA). FTA solution (>98% HPLC purity; cat. no. L28-110506; Jiangxi Tiangong Herbal Technology Co., Ltd.) was prepared at a concentration of 1 mg/ml in sterile water. The materials under study were endotoxin-free.

Animals. Male BALB/c mice (18±2 g, 6-8 weeks old) were purchased from Hunan Slack King Laboratory Animal Co., Ltd. The mice were housed in a room maintained at 24±1˚C with 40-80% humidity. All animals received food and water *ad libitum*. Mice were housed for 2-3 days to adapt to the environment before the experiments. The experimental procedures were approved by the Commission of Nanchang University for Ethics of Experiments on Animals and were conducted in accordance with international standards. All surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize animal suffering.

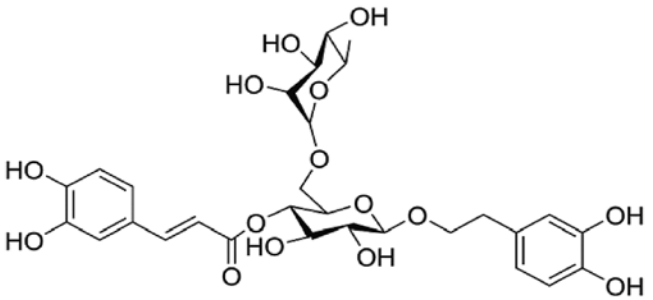

Figure 1. Chemical structure of forsythoside A (FTA).

Leukocyte isolation. Two to four spleens were converted into single-cell suspensions by squeezing through a 74-μm nylon net with the rough end of a 5-ml syringe plunger. The single-cell suspensions were filtered using a 37-μm nylon net. Then, the cells were spun down in a 15-ml Falcon tube (10 min, 1,500 rpm) and the supernatant was discarded. The pellets were resuspended in 1 ml of warm RPMI-1640 medium, layered above 2 ml of lymphocyte separation medium (LSM; Solarbio, Beijing, China) and centrifuged for 20 min at 20˚C at 2,000 rpm. The top layer of clear plasma was aspirated to within 2-3 mm above the lymphocyte layer and discarded. Next, the lymphocyte layer were aspirated and diluted with warm RPMI-1640 medium into a new centrifuge tube and centrifuged for 10 min at 1,000 rpm twice. Finally, the cells were resuspended in warm RPMI-1640 medium.

Cell culture and cell groups. RAW264.7 cells (Department of Gastroenterology, Jiangxi, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin, 100 mg/ml of streptomycin). RAW264.7 cells were divided into 6 groups: i) control group, incubated without any treatment; ii) LPS group, incubated with LPS (100 ng/ml) for 12 h; (iii-v) FTA groups: preconditioned by incubation with FTA (20, 80 or 320 μg/ml) for 2 h before the addition of LPS (100 ng/ml) and incubation for 12 h; vi) PMB group, preconditioned with PMB (10 μg/ml) before the addition of LPS (100 ng/ml) and incubation for 12 h.

Isolated mouse lymphocytes were suspended in 10% FBS and RPMI-1640 medium at a concentration of $1x10^6$ cells/ml and plated in 6-well multiplates prior to the addition of ConA (final concentration, 5 μg/ml) and IL-2 (final concentration, 100 ng/ml) (both from Sigma). Cell cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Lymphocytes were divided into 6 groups and treated as

described above for RAW264.7 cells, except with longer (48 h) LPS stimulation.

Cell viability assay. Cell viability was assessed using the MTT assay. All cells were cultured in 96-well plates in an incubator at 37˚C and 5% CO_2, with RAW264.7 cells cultured at a density of 10^5 cells/well for 12 h and lymphocytes cultured at 10^6 cells/well for 2 h. Cells were washed with fresh medium prior to incubation with a range of FTA concentrations (20, 80 or 320 μg/ml) or PMB (10 μg/ml) for 2 h. The medium was discarded and LPS (100 ng/ml) was added to the incubation mixture. RAW264.7 cells and lymphocytes were incubated with LPS for 12 and 48 h, respectively. Cells were washed and 20 μl of MTT (5 mg/ml) was added, followed by incubation for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and the amount of formazan salt was determined by measuring the absorbance at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Data are expressed as means ± SD from at least 3 independent experiments.

ELISA for quantification of TNF-α, IL-10 and TGF-β1 levels. Expression of TNF-α in RAW264.7 cell culture supernatants was quantified using a commercially available ELISA kit, according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). IL-10 and TGF-β1 levels were determined in supernatants obtained from the lymphocyte culture using ELISA kits (Westang Co., Ltd., Shanghai, China). The absorbance was read at 450 nm using a microplate reader (Bio-Rad). The levels of cytokines were calculated using standard curves prepared by analyzing a range of concentrations of purified recombinant TNF-α, IL-10 and TGF-β1.

Flow cytometric analysis. Primary lymphocytes in each group was collected and washed twice with phosphate-buffered saline (PBS) and further divided into the isotype control group and experimental group. Cells in the isotype control group were stained with Armenian hamster IgG isotype control PE-cyanine5, rat IgG1 isotype control PE, and rat IgG2a isotype control FITC (all from eBioscience), while the experimental group was stained with anti-mouse CD3e PE-Cy5, anti-mouse CD4 FITC, and anti-mouse CD25 PE (eBioscience) at the concentrations recommended by the manufacturer for 15 min in the dark at room temperature. Red blood cell lysis buffer (1 ml) was added to each sample of collected primary lymphocytes and incubated for 10 min in the dark at room temperature. Finally, the cells were washed with PBS and fixed with RPMI-1640 medium prior to analysis in a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blotting for TLR4, MyD88, NF-κB and Foxp3. RAW264.7 cells and lymphocytes were plated onto 6 60-mm plastic dishes and incubated as described above. The expression levels of TLR4, NF-κB and MyD88 in RAW264.7 cells and Foxp3 in lymphocytes were determined by western blot analysis, using β-actin expression as a reference. Briefly, after drug treatment, the cells were lysed with ice-cold RIPA buffer (Solarbio), and the protein content of the lysates was measured using the bicinchoninic acid (BCA) method. Equal amounts of cellular proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter membrane. After blocking with 5% non-fat milk powder, the membranes were incubated with respective rabbit anti-mouse polyclonal antibodies [TLR4 (ab13556), MyD88 (ab2068), Foxp3 (ab54501) or NF-κB (ab16502)] and murine monoclonal antibodies [β-actin (ab8226)] (both from Abcam, Cambridge, MA, USA) at 4˚C overnight. After washing in TBST (3 washes of 10 min each), the membranes were incubated with peroxidase-conjugated Affinipure goat anti-rabbit (ZB-2301) or mouse (ZB-2305) immunoglobulin G antibodies (ZSGB-BIO, Beijing, China) for 1 h at room temperature and visualized with enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following exposure to X-ray film. The relative band intensity was quantified by Quantity One software v4.62 (Bio-Rad) to determine the protein levels.

Quantitative (real-time) PCR. Using EZN Total RNA Kit II (Omega Bio-Teck, Doraville, GA, USA), the total RNA was extracted from RAW264.7 cells and lymphocytes, according to the maufacturer's instructions. cDNA was produced using PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology, Dalian, China). Real-time polymerase chain reaction was performed on an ABI-Prism StepOne using SYBR *Premix Ex Taq* II (Takara Biotechnology). The primers used were 5'-TTTATTCAGAGCCGTTGG-3' and 5'-AGTTGC CGTTTCTTGTTG-3' for mouse TLR4; 5'-ACTCGCAGTTT GTTGGATG-3' and 5'-ACTCGCAGTTTGTTGGATG-3' for mouse MyD88; 5'-CTCATGATAGTGCCTGTGTCCTCAA-3' and 5'-AGGGCCAGCATAGGTGCAAG-3' for mouse Foxp3; 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and 5'-ATGGA GCCACCGATCCACA-3' for mouse β-actin. The PCR amplification profiles consisted of denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing at 62˚C for 34 sec. All amplification reactions for each sample were carried out in triplicate, and the relative expression values were normalized to the expression value of mouse β-actin.

Data processing. Statistical analysis was performed with IBM SPSS Statistics software (version 19.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as mean ± SEM. The differences between the data sets were assesses by one-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK)-q test. P-value <0.05 was considered to indicate a statisical significant result.

Results

FTA increases the viability of LPS-treated RAW264.7 cells and lymphocytes. RAW264.7 cells and lymphocytes treated with LPS alone exhibited severe cell damage and reduced cell viability compared to the untreated control cells (P<0.01) (Fig. 2). Treatment with FTA dose-dependently reduced LPS-induced damage in the RAW264.7 cells and lymphocytes (Fig. 2).

FTA reduces cytokine production

TNF-α. The accumulation of TNF-α was measured to assess the effect of FTA treatment on this proinflammatory cytokine. LPS-treated RAW264.7 cells exhibited markedly increased

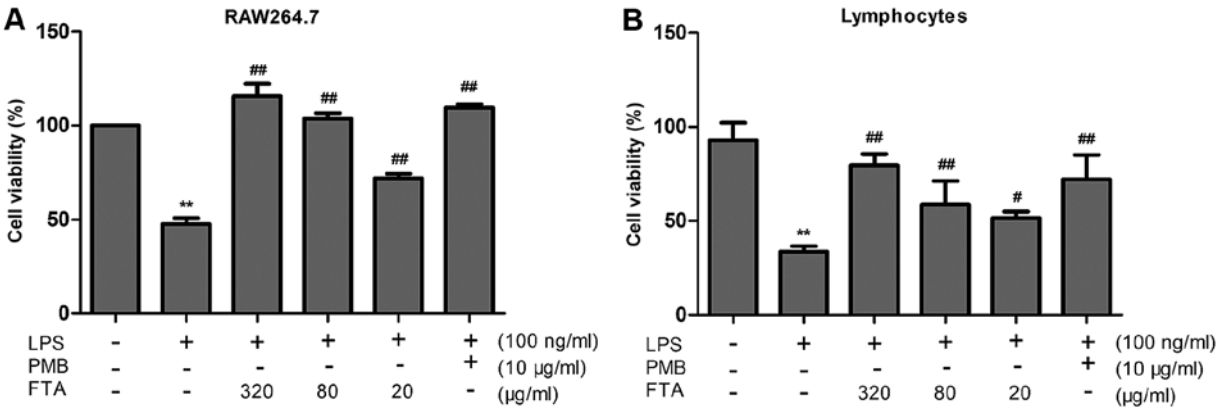

Figure 2. Effect of forsythoside A (FTA) on the viability of cells. RAW264.7 cells (A) and lymphocytes (B) were stimulated with lipopolysaccharide (LPS, 100 ng/ml, 12 or 48 h, respectively) in the absence or presence of pretreatment with polymyxin B (PMB, 10 μg/ml, 2 h) or FTA (320, 80 and 20 μg/ml, 2 h). MTT cell-viability assays were conducted. PMB was used as the positive control. Values are expressed as the mean ± SEM of six (A) or eight (B) independent experiments. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. the LPS-treated group.

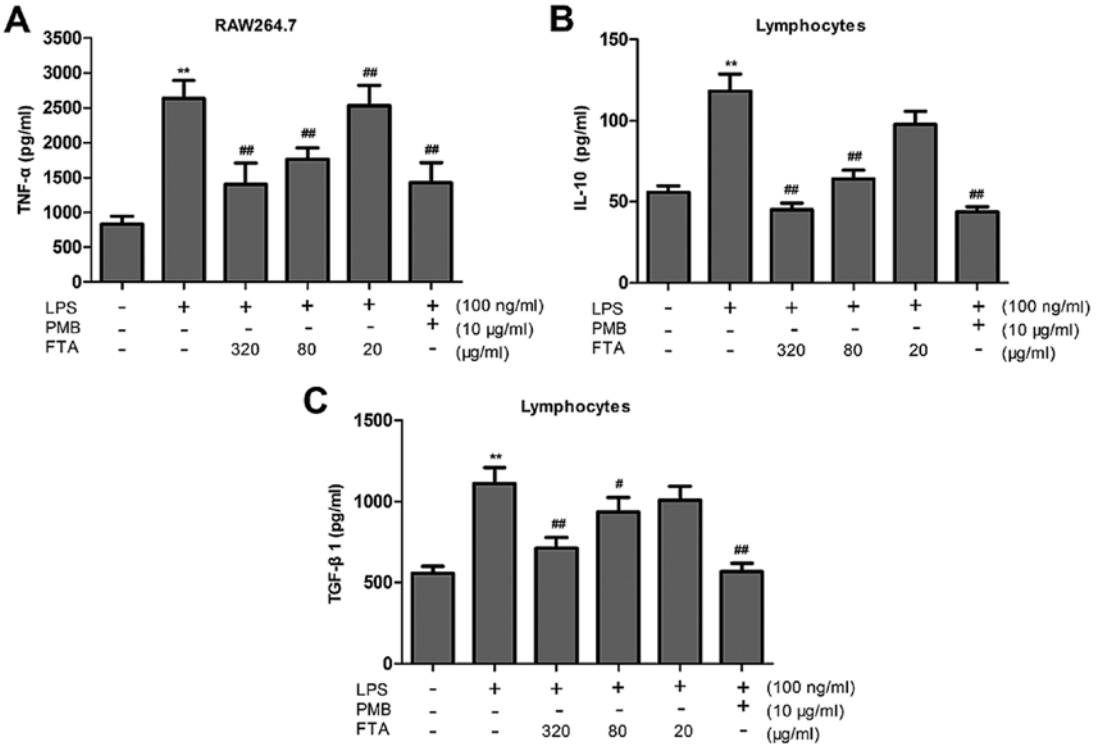

Figure 3. Forsythoside A (FTA) inhibits lipopolysaccharide (LPS)-induced release of cytokines. The levels of TNF-α were measured in RAW264.7 cell culture (A). IL-10 (B) and TGF-β1 (C) levels were determined in lymphocyte culture. Values are expressed as the mean ± SEM of eight (A) or three (B and C) independent experiments. **P<0.01 and *P<0.05 vs. the control group; $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. the LPS-treated group.

cytokine production compared to the control group, while FTA treatment significantly inhibited the LPS-induced increase in TNF-α concentration in a dose-dependent manner (Fig. 3A).

IL-10 and TGF-β1. We examined the effect of FTA on the production of proinflammatory cytokines IL-10 and TGF-β1. Lymphocytes were pretreated with FTA for 2 h and levels of IL-10 and TGF-β1 were measured in culture media by ELISA. IL-10 and TGF-β1 were found to be upregulated in the LPS-stimulated lymphocytes, with the pretreatment with FTA eliciting suppression of the LPS-induced increase (Fig. 3B and C). These results suggest that the immunomodulatory effect of FTA may be mediated through the inhibition of IL-10 and TGF-β1 production.

FTA inhibits LPS-induced activation of TLR4/MyD88/NF-κB signaling. To elucidate the mechanisms underlying the inhibition of LPS-induced production of proinflammatory cytokines, we investigated the expression levels of TLR4, MyD88 and NF-κB protein, and TLR4 and MyD88 mRNA in RAW264.7 cells. LPS binds TLR4, leading to the activation of MyD88-dependent and MyD88-independent signaling pathways. NF-κB is activated within the MyD88-dependent pathway and has been implicated in the regulation of the TNF-α promoter (23). The expression of proteins involved in the TLR4 signaling pathway was increased in the RAW264.7 cells after LPS administration (Fig. 4). Incubation with FTA

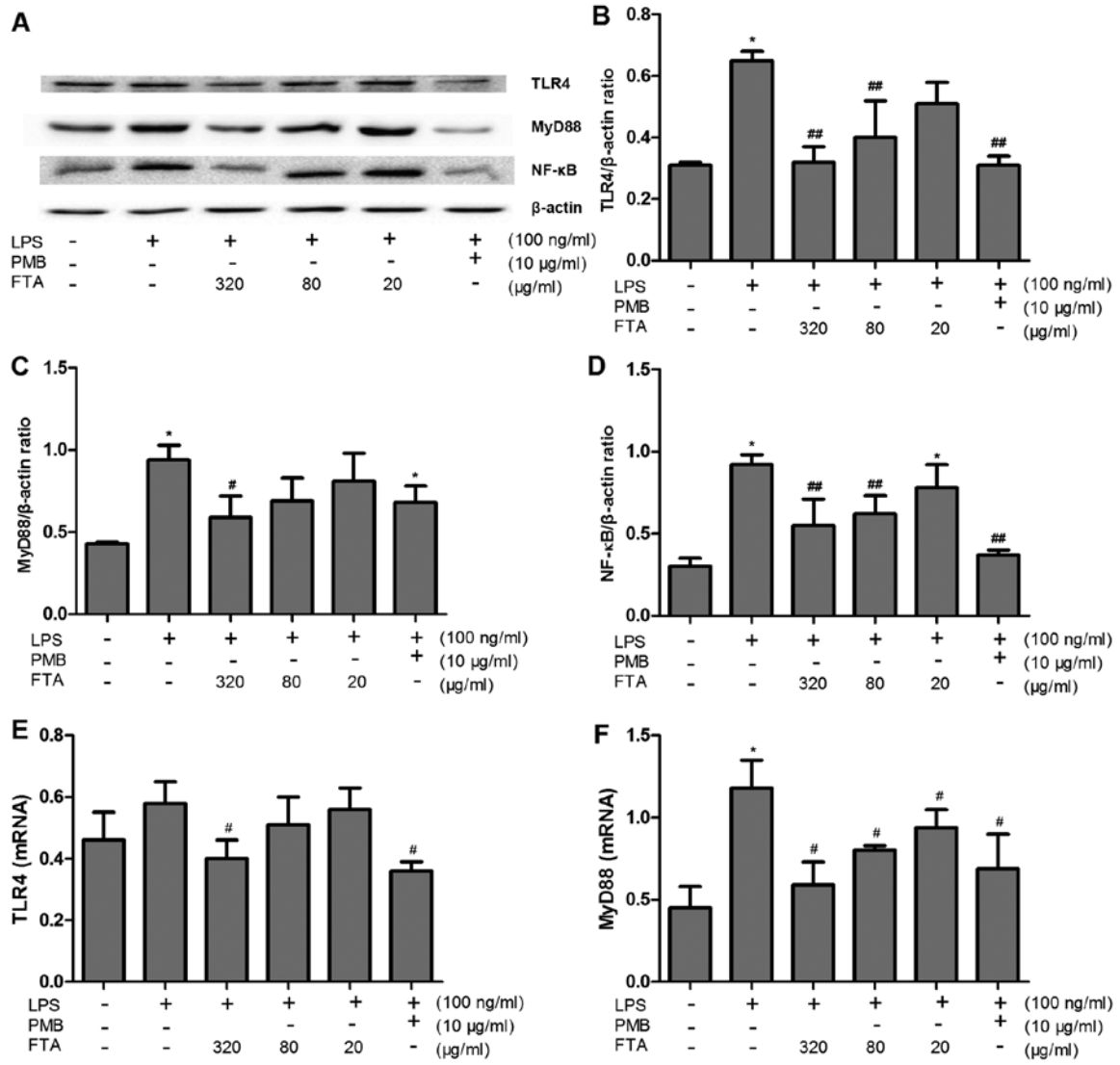

Figure 4. Forsythoside A (FTA) inhibits lipopolysaccharide (LPS)/TLR4 signaling in a dose-dependent manner. RAW264.7 cells were stimulated with LPS (100 ng/ml, 12 h) in the absence or presence of pretreatment polymyxin B (PMB, 10 μg/ml, 2 h) or FTA (320, 80 and 20 μg/ml, 2 h). The expression levels of Toll-like receptor 4 (TLR4) (A and B), MyD88 (A and C), and nuclear factor-κB (NF-κB) (A and D) protein were assayed by western blot analysis. The expression levels of TLR4 (E) and MyD88 (F) mRNA were assayed by quantitative PCR. β-actin was used as a loading control. Values are mean ± SEM of three independent experiments. **P<0.01 and *P<0.05 vs. the control group; $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. the LPS-treated group.

significantly inhibited the effect of LPS in a concentration dependent-manner. These results suggest that FTA inhibits the TLR4 signaling pathway and thereby protects macrophages from LPS stimulation.

FTA decreases the Treg percentage and the expression levels of Foxp3 protein and mRNA

FTA decreases Treg percentage. LPS induced a marked increase in the relative percentage of $CD4^+CD25^+/CD4^+$ T lymphocytes (P<0.01), which was significantly attenuated by treatment with FTA (80 and 320 μg/ml) (Fig. 5G). FTA treatment decreased LPS-induced changes in the relative percentage of Tregs in a dose dependent manner. These findings are in agreement with the results of flow cytometry (Fig. 5A-F). The *in vitro* results, therefore, suggest that FTA elicits immunomodulatory effects.

FTA decreases the expression levels of Foxp3 protein and mRNA. The expression levels of Foxp3 protein and mRNA in the LPS-treated cells were markedly increased compared to these levels in the control group (P<0.01) (Fig. 6). However, FTA treatment significantly decreased levels of Foxp3 protein and mRNA in the LPS-treated cells in a dose-dependent manner (P<0.01 or P<0.05).

Discussion

Plants used in traditional medicine are rich in physiologically active ingredients. There is a trend in recent pharmacological research to seek novel therapeutic agents on the basis of traditional Chinese medicine. Of the 80% of pharmaceuticals that are derived from plants, few are currently used as anti-microbial agents (24). LPS are ubiquitous and act as powerful stimulators of the immune system. Recently, a number of research groups have sought to develop treatments that are effective against LPS-mediated toxic effects by investigating the ingredients of traditional medicinal plants. The decoction of *Forsythia* was reported to elicit anti-inflammatory

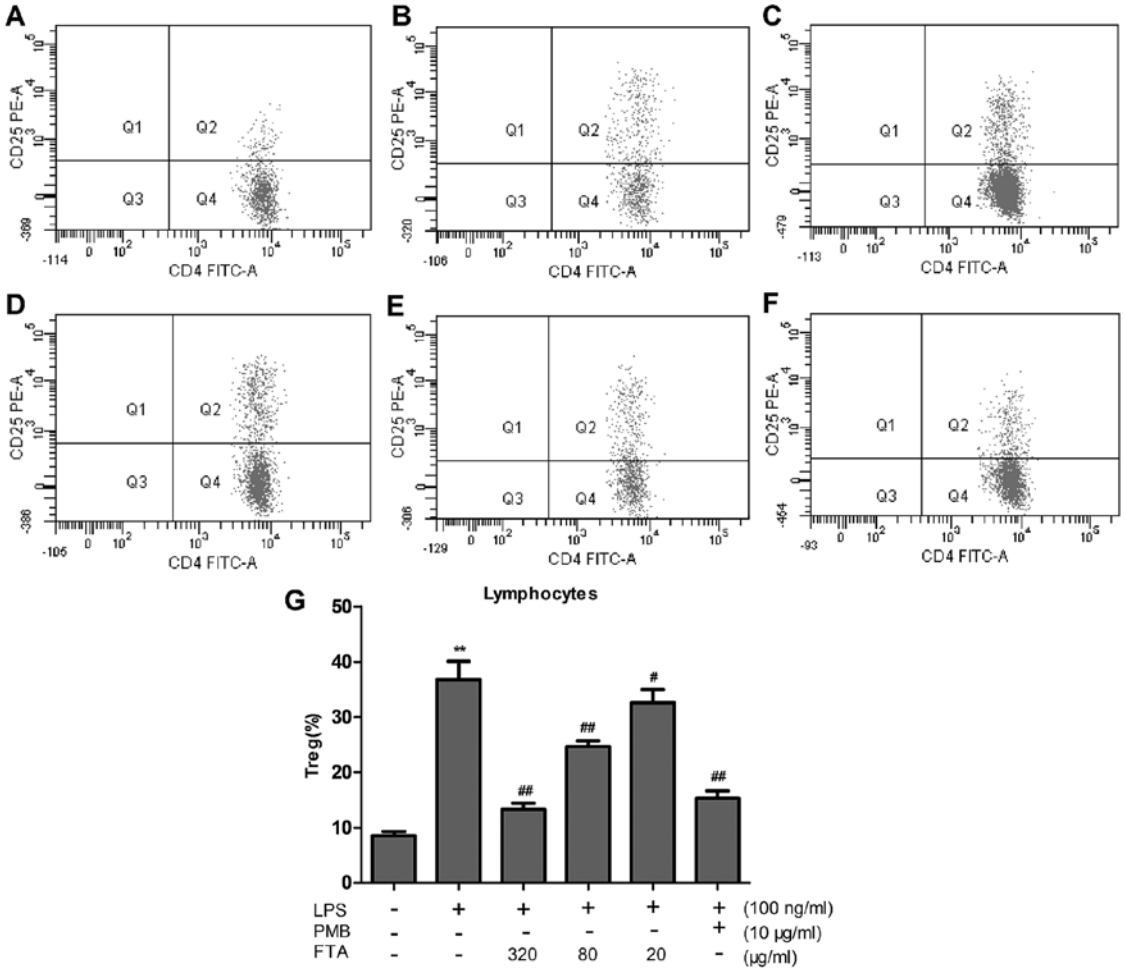

Figure 5. Forsythoside A (FTA) decreases the Treg percentage in a dose-dependent manner. Lymphocytes were stimulated with lipopolysaccharides (LPS) (100 ng/ml, 48 h) in the absence or presence of pretreatment with polymyxin B (PMB, 10 μg/ml, 2 h) or FTA (320, 80 and 20 μg/ml, 2 h). The Treg percentage in total lymphocytes was quantified by flow cytometric analysis of (A) control-treated lymphocytes, (B) lymphocytes treated with LPS alone, (C) lymphocytes treated with LPS with FTA pretreatment (320 μg/ml), (D) lymphocytes treated with LPS with FTA pretreatment (80 μg/ml), and (E) lymphocytes treated with LPS with FTA pretreatment (20 μg/ml), (F) lymphocytes treated with LPS with pretreatment with PMB (10 μg/ml). (G) Histogram showing flow cytometry results. Values are expressed as the mean ± SEM of three independent experiments. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. the LPS-treated group.

effects and, importantly, to ameliorate the adverse effects of LPS (25). In the present study, we demonstrated that FTA, the main antimicrobial constituent in *Forsythia* (26), exerts significant anti-endotoxin activity in LPS-treated mice and LPS-stimulated cells. The effect appears to be associated with the inhibition of the LPS-TLR4 signaling pathway and decreased number of Tregs.

PMB, a compound that exhibits strong affinity for LPS, is generally perceived to be an endotoxin antagonist. A low dose of PMB (50,000 U/day) was reported to decrease plasma endotoxin levels without eliciting any significant side effects (27). PMB-immobilized fiber column hemoperfusion has been used for treating septic shock, despite its potential to induce severe nephrotoxicity and neurotoxicity (28). Due to its anti-LPS efficacy, PMB was used as a positive control in our present study.

TLR, a receptor family closely related to the innate immunity, can recognize pathogen-associated molecular patterns (PAMP) and regulate innate and acquired immunity. TLR4 is the first TLR identified in humans, and is considered to be the most important receptor involved in the effects of LPS. Tissue damage and infection lead to the recognition of bacterial lipoprotein and bacterial LPS by TLR4, initiating the signal transduction cascade and inducing the release of endogenous mediators, thereby stimulating the inflammatory response. The amplification cascade of inflammatory factors can aggravate infection and cause further tissue damage (29). Cell viability was significantly decreased compared to the vehicle-treated control group (P<0.05). Our findings, therefore, confirm the presence of adverse effects of LPS on cellular function. However, therapeutic agents have been shown to significantly reduce LPS-induced pathological changes in a dose-dependent manner. In our present study, we confirmed that FTA can ameliorate the effect of LPS on cell proliferation at the cellular level. Further study showed that levels of TLR4, MyD88, NF-κB and TNF-α protein in the FTA groups to be significantly and dose-dependently reduced (P<0.05), compared to those measured in the LPS groups. Through the effect involving several intracellular signaling molecules, LPS stimulates TLR4 signaling pathway downstream of the receptor, affecting both MyD88-dependent

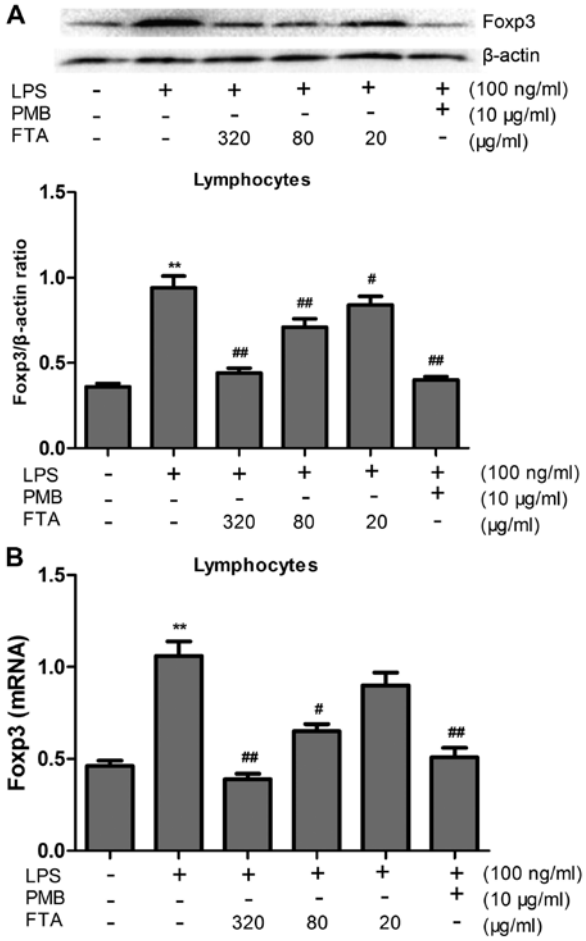

Figure 6. (A and B) Forsythoside A (FTA) decreases Foxp3 protein and mRNA expression in a dose-dependent manner. Lymphocytes were stimulated with lipopolysaccharides (LPS) (100 ng/ml, 48 h) in the absence or presence of pretreatment with polymyxin B (PMB, 10 μg/ml, 2 h) or FTA (320, 80 and 20 μg/ml, 2 h). Foxp3 protein level was assayed by western blot analysis. Foxp3 mRNA was assayed by quantitative real-time PCR. β-actin was used as a loading control. Values are expressed as the mean ± SEM of three independent experiments. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. the LPS-treated group.

and MyD88-independent (TRIF-dependent) pathways (30). Within the MyD88-dependent pathway, infection and cell damage activate signaling cascades involving inflammatory cytokines. Regardless of the signaling pathway, intracellular factors such as NF-κB and IRF3 are activated. NF-κB is an important inducible transcription factor which can regulate the expression of cytokines such as TNF-α, IL-6 and IL-8, along with other cytokines involved in the inflammatory and immune response, cell proliferation, tissue differentiation and apoptosis (31,32). While TNF-α is important for the normal inflammatory response to infection, inappropriate or excessive production can be harmful (33). TNF-α acts through the TNF-α receptor I or TNF-α receptor II to induce apoptosis, regulate cell survival, or modulate inflammation (34). The successful use of TNF blockade in the management of chronic inflammatory diseases highlights the physiological role of TNF in sepsis (33). FTA may, therefore, inhibit inflammatory factors TNF-α and NF-κB through blockade of the LPS/TLR4 signaling pathway to elicit anti-endotoxin effects.

Caramalho *et al* previously reported that Tregs interact with LPS since they selectively express TLR4, thereby supporting their survival and proliferation, while also enhancing their immunosuppressive function (35). The present study showed that FTA suppresses LPS-mediated induction of the TLR4 pathway. Therefore, we propose that the protective effect of FTA may be elicited by regulation of Tregs.

Tregs elicit immunomodulatory effects and play a pivotal role in maintenance of the immune balance. $CD4^+CD25^+$ Tregs inhibit $CD4^+$ T cell proliferation (36). In infection, Tregs mediate the responses of T cells to pathogens and their activation of inflammatory response to tissue damage (37). Our data showed a significantly lower survival rate of LPS-stimulated lymphocytes, compared to the control group, while the relative presence of Tregs in culture was significantly higher (P<0.01). These findings indicate that the immunosuppressive ability of LPS-stimulated lymphocytes is inhibited, and confirm that LPS adversely affects Treg function. The effects of LPS may be related to the excessive activation of Tregs, resulting in inhibition of $CD4^+CD25^-$ cell proliferation. Experiments evaluating the effects of FTA intervention showed that FTA can significantly increase the survival rate of LPS-stimulated lymphocytes and decrease the relative presence of Tregs in a dose-dependent manner (P<0.05). In comparing cells treated with a high dose of FTA with the control group, no obvious difference was found (P>0.05), suggesting that the cytoprotective effect of FTA may involve the inhibition of Treg activation to correct the LPS-induced inhibition of cell immunity. Foxp3 is widely recognized to act as a master switch and transcription factor for Treg development and function (38), and is the most specific biomarker for Treg activation (39). Deletion of the Foxp3 gene was found to eliminate the immunosuppressive activity of $CD4^+CD25^+$ Tregs, while the ectopic expression of Foxp3 in $CD25^-$ Tregs conferred immunosuppressive activity to the cells (40). Human Foxp3 gene mutation has been linked with immunological dysfunction, inflammatory bowel disease (IBD), X-linked syndrome and allergic dermatitis (41). In light of the significance of Foxp3, we evaluated the expression of this protein in our experiments. In LPS-stimulated groups, Foxp3 expression was markedly increased, with this increase being significantly suppressed in FTA groups. These findings indicate that FTA may inhibit Treg activity by inhibiting Foxp3. IL-10 and TGF-β1 are involved in the regulation of Foxp3 expression and the secretion of IL-10 and TGF-β1 is one of the ways in which Tregs suppress antigen-driven response of $CD4^+CD25^-$ cells (42-45). The two aspects contribute to the immunosuppressive activity of $CD4^+CD25^+$ Tregs. In this study, FTA intervention could significantly inhibit IL-10 and TGF-β1, which may be one of the mechanisms underlying the observed downregulation of Foxp3.

In conclusion, we validated the therapeutic potential of FTA for endotoxin-induced diseases. Furthermore, we identified the blockade of the LPS/TLR4 signaling pathway and inhibition of Tregs as putative mechanisms underlying the protective action of FTA. While this study demonstrates the potential for clinical efficacy of FTA, it should be noted that the clinical effects may differ from our experimental results due to the involvement of bacteria other than the LPS-producing species in the clinical setting. Further studies are, therefore, warranted to extend our experimental findings into the clinical setting.

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