Original Research

Protective effect of astaxanthin on tuberculosis-associated inflammatory lung injury

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Impact Statement

How to protect pulmonary tuberculosis (PTB)–related lung damage is a problem that this research hopes to solve. Astaxanthin (ASTA) is a natural compound with great potential for protection against inflammatory damage to lung tissue. In murine lung epithelial (MLE)-12 cells, lipopolysaccharide (LPS) could induce apoptosis via regulating the nuclear factor kappa B (NF-κB) signaling pathway. Thus, we used LPS to establish an *in vitro* inflammatory injury model of lung epithelial cells. We found that ASTA can reduce lung inflammation and protect lung tissue from inflammatory damage.

Abstract

Mycobacterium tuberculosis (MTB) invades the lungs and is the key cause of tuberculosis (TB). MTB induces immune overreaction and inflammatory damage to lung tissue. There is a lack of protective drugs against pulmonary inflammatory damage. Herein, the protective roles and mechanisms of Astaxanthin (ASTA), a natural compound, in inflammatory injured lung epithelial cells were investigated. Lipopolysaccharide (LPS) was used to establish inflammatory injury model in the murine lung epithelial (MLE)-12 cells. Cell counting kit-8 was used for screening of compound concentrations. Cell proliferation was observed real-time with a high content analysis system. Flow cytometry assessed apoptosis. The changes of apoptotic proteins and key proteins in nuclear factor kappa-B (NF-κB) pathway were measured with the western blot. LPS was used to establish an animal model of pulmonary injury. The pathological changes and degree of inflammatory injury in lung tissue were observed with hematoxylin and eosin (HE) staining. The levels of inflammatory mediators were detected with enzyme-linked immunosorbent assay. The results showed that ASTA reduced lung inflammation and attenuated inflammatory damage in lung tissues. ASTA reduced apoptosis stimulated by LPS through suppressing the NF-κB pathway in MLE-12 cells. We believe that ASTA may have great potential for protection against inflammatory damage to lung tissue.

Keywords: Tuberculosis, inflammatory lung injury, Astaxanthin, NF-κB signaling pathway, apoptosis, flow cytometry

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Introduction

Tuberculosis (TB), a leading factor causing infectious disease–related deaths, is a respiratory system disease resulted from Mycobacterium tuberculosis (MTB), and the lesions mainly occur in lung tissue, trachea, bronchi, and so on.[1,2] Respiratory symptoms of TB patients include sputum, cough, chest pain, hemoptysis, and varying degrees of dyspnea or chest tightness.[3] The main role of drug treatment is to shorten the infectious period, reduce mortality, infection rate, and morbidity. For example, isoniazid, rifampin, and other drugs have been widely used to treat TB, and achieved good curative effect.[4] However, there is persistent pulmonary dysfunction from mild pulmonary dysfunction to severe dyspnea in most TB survivors, increasing the risk of death from respiratory causes.[5]

It is reported that TB-induced dysfunction is associated with inflammatory damage to lung tissue.[6] Tumor necrosis factor-α (TNF-α), a key mediator of host immune responses to TB, can also promote apoptosis.[7] TNF-α may promote necrosis of normal cells via inducing mitochondrial reactive oxygen species.[8,9] The hyperinflammation and cell death result in lung tissue damage and cavitation. In macrophages infected with MTB, TNF induces mitochondrial reactive oxygen species via receptor interacting protein-1,3 dependent pathways.[10,11] In the inflammatory environment, TNF-α

mainly affects the physiological state of cells via the NF-κB (nuclear factor kappa-B) pathway.[12] Therefore, NF-κB pathway may potentiate tissue destruction during TB.

Astaxanthin (ASTA) is a red carotenoid most commonly found in marine and aquatic animals.[13] ASTA can reduce the production of reactive nitrogen free radicals and hinder the expression of inflammatory cytokines including interleukin (IL)-6 and IL-1β.[14] However, whether ASTA can reduce TB-related inflammatory damage has not been reported. In this study, we will explore whether ASTA has a protective effect on TB-related inflammatory injury based on the NF-κB signaling pathway.

Materials and methods

Cells

MLE-12, the mouse alveolar epithelial cell line, was cultured in 1640 medium containing 10% fetal bovine serum at 37°C with 5% CO_2. The cell was from OTWO Biotech (Shenzhen, China).

Reagents

MedChemExpress (New Jersey, USA) provided ASTA (purity > 95%) and pyrrolidinedithiocarbamate ammonium (PDTC) (purity > 95%). YEASEN (Shanghai, China) provided the dimethyl sulfoxide, ECL chemiluminescence kit, and cell counting kit-8 (CCK-8). RPMI medium modified (1640) (Hyclone, MA, USA) and fetal bovine serum (ExCell Bio, Shanghai, China) were used for cell culture. Except anti-caspase 9 antibody (HUABIO, Hangzhou, China), antibodies against NF-κB/p65, p-NF-κB/p-p65, inhibitor of nuclear factor kappa-B kinase subunit beta (Ikkβ), Ika, p-Ika, caspase 3, and glyceraldehyde 3-phosphate dehydrogenas (GAPDH) as well as goat antirabbit immunoglobulin G (IgG) (H + L) labeled with horseradish peroxidase (HRP) were provided by ImmunoWay (Plano, TX, USA). BD (Franklin Lake, New Jersey, USA) provided Annexin V-FITC apoptosis kit.

CCK-8 assay

After culturing (1×10^4 cells/well; 96-well plate) for 4 h, different concentrations of ASTA or PDTC were added to MLE-12 cells for 24 h incubation. Then, CCK-8 solution (10 μL) was introduced for 1 h incubation. The Bio-Rad microplate reader (Hercules, CA, USA) evaluated OD450.

Dynamic cell proliferation and monitoring

The 96-well plate was used for MLE-12 cell culture (1×10^3 cells/well). After overnight treatment with different concentrations of compounds, the 24 h dynamic monitoring of real-time cell proliferation, and differentiation were performed with a high content analysis system.

Cell apoptosis detection

After 24 h culture (1×10^5 cells/well; 6-well plate), ASTA and PDTC were added to MLE-12 cells and incubated for 24 h. Subsequently, the Annexin V-FITC/PI apoptosis kit and FACSCelesta™ (BD) detected apoptosis.

Western blot analysis

After ASTA and PDTC treatment, MLE-12 cells were subjected to protein extraction after collection. Proteins were transferred to nitrocellulose membranes after separation. Following blocking, the caspase 3, caspase 9, Bax, Bcl-2, p65, p-p65, Ikkβ, Ikα, p-Ikα, and GAPDH primary antibodies were incubated at 4°C overnight. Then, the 2-h room temperature incubation with goat antirabbit IgG was conducted. After visualization with ChemiDoc™ XRS+ (Bio-Rad), the band intensities were quantified by Image J software.

Animals

C57BL/6 male mice (4-week-old, 16–19 g, $n = 32$) were purchased from Gansu University of Traditional Chinese Medicine. They were kept under 12 h dark/light cycle, with free access to water and food and in a temperature-controlled room. The surgeries were conducted under anesthesia with sodium pentobarbital. The Committee Ethics on Animal Experiments of Gansu University of Chinese medicine (no. 2020-232) approved all animal experimental procedures. All experiments followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The suffering was minimized with every effort.

Inflammatory lung injury model

Control group ($n = 8$), lipopolysaccharide (LPS) (Solarbio, Beijing, China) group ($n = 8$), ASTA treatment group ($n = 8$), and PDTC treatment group ($n = 8$) were set up. The control group received sterile saline. The mice were inoculated with LPS in the abdominal cavity (25 mg/kg). After 12 h, the animals were euthanized and the lungs were harvested to make paraffin sections for hematoxylin and eosin (HE) staining.

Enzyme-linked immunosorbent assay

The lung tissue of the mouse was crushed and ground. After centrifugation, the levels of IL-6, TNF-α, NO, and IL-10 in the supernatant were measured with corresponding enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemicals, Ann Arbor, MI, USA).

Statistical analysis

SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. At least three independent experiments were performed. Data are described as mean value ± SD. Student's *t*-test and one-way analysis of variance (ANOVA) compared the data. Statistical significance was determined if $P < 0.05$.

Results

ASTA promotes MLE-12 cell proliferation

The optimal concentration of ASTA (Figure 1(A)) and PDTC was determined by measuring cell viability with CCK-8 assay. Treatment with ASTA and PDTC was performed for 24 h before CCK-8 assay. We found that cell viability was increased by the treatment of ASTA at the concentration of more than 15 μmol/L for 24 h (Figure 1(B)) or by the treatment of PDTC at the concentration of more than 10 μmol/L for 24 h

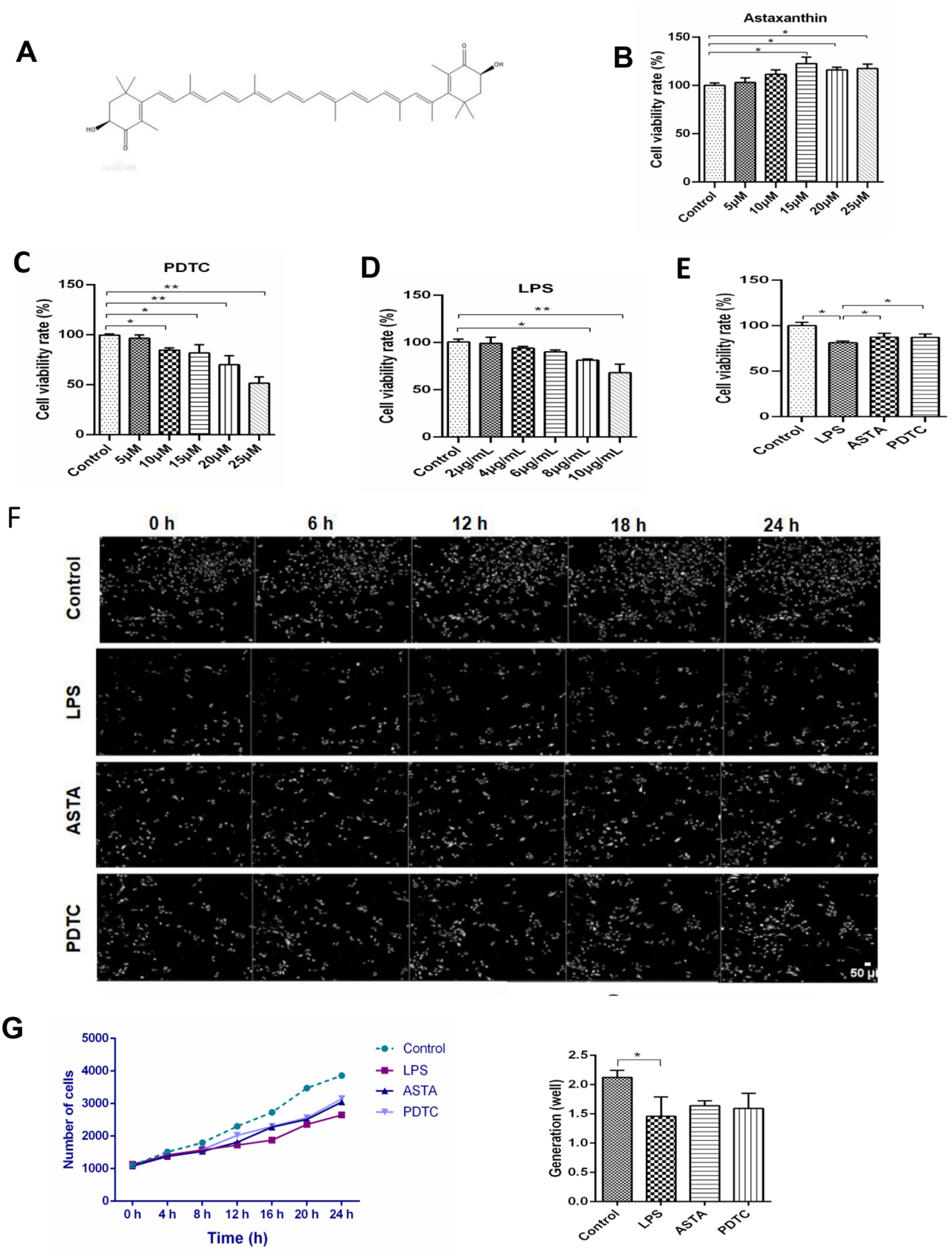


Figure 1. ASTA maintains MLE-12 cell proliferation. (A) Molecular structure of ASTA. (B to D) MLE-12 cells were intervened for 24 h with indicated doses of ASTA, LPS, and PDTC for 24 h, and CCK-8 assessed cell viability, $n=5$. (E) MLE-12 cells were treated with indicated concentrations of ASTA, LPS, and PDTC, and CCK-8 assessed cell viability, $n=5$. (F) Real-time monitoring of MLE-12 cell proliferation within 24 h (scale bar = 50 μm). (G) Differentiation generations of MLE-12 within 24 h.

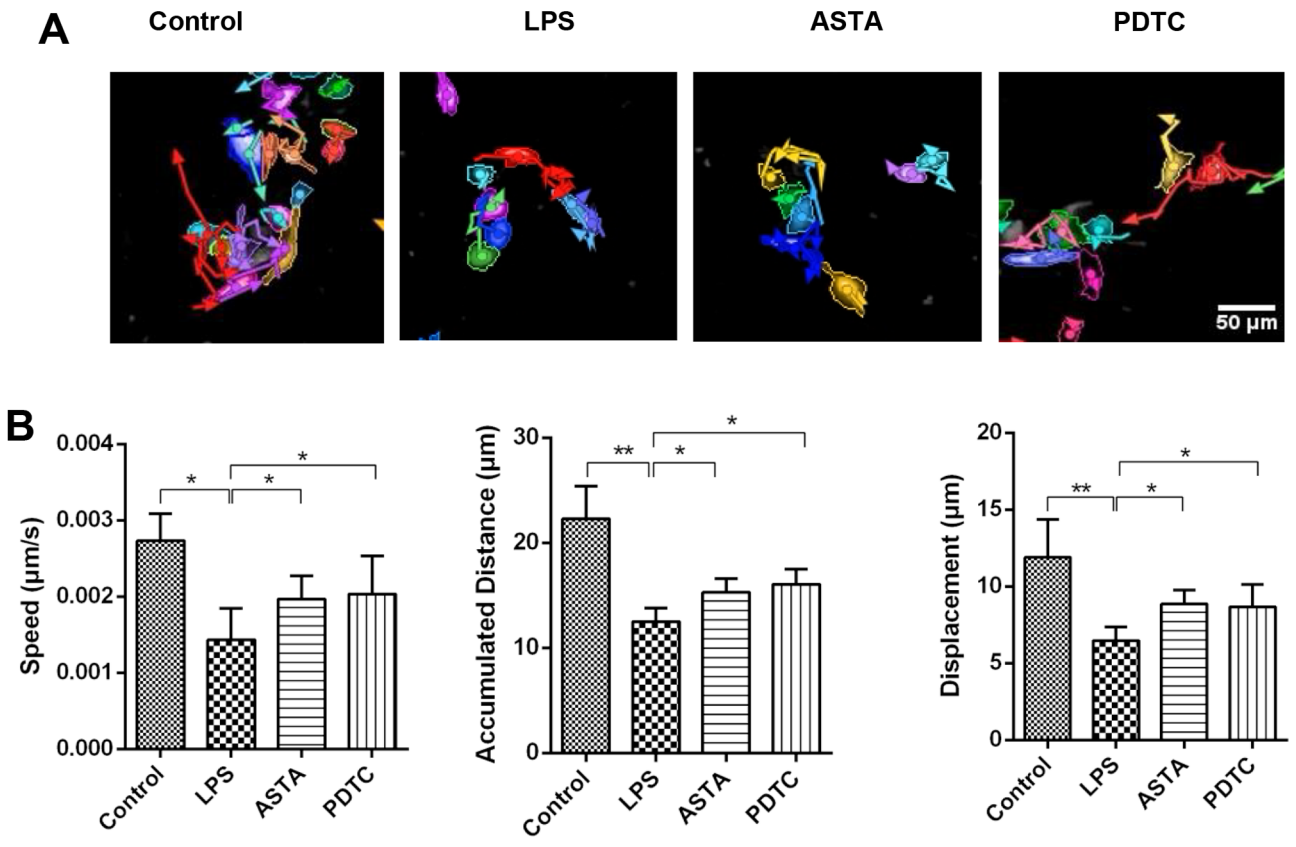


Figure 2. ASTA maintains MLE-12 cell motility. (A) Time monitoring of cell movement trajectories (scale bar = 50 μm). (B to D) The speed, distance, and accumulated distance of cell movement in 24 h. Data are described with mean value ± SD.
Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$.

(Figure 1(C)). Thus, the optimal concentration of ASTA and PDTC was determined at 1 and 5 μmol/L, respectively.

We found that independent ASTA has obvious proliferation effects on MLE-12 cells. But whether ASTA still has a promoting effect in an inflammatory environment is unclear. We modeled TB-associated inflammatory injury of lung epithelial cells by LPS. We observed that LPS at the concentration of more than 8 μg/L decreased cell proliferation (Figure 1(D)). Thus, the concentration of LPS was determined at 8 μg/L. We further found that both ASTA and PDTC could reverse LPS-induced suppression of proliferation in MLE-12 cells.

Cell proliferation after treatment with ASTA, PDTC, and LPS was assessed on a high content analysis system. We revealed that LPS suppressed the differentiation and proliferation of MLE-12 cells, while both ASTA and PDTC reversed these effects (Figure 1(F) and (G)).

ASTA promotes the motility of MLE-12 cells

The motility cells can show their viability. Monitoring with high content analysis system showed that ASTA significantly promoted the range and path of MLE-12 cell activity within 24 h (Figure 2(A)). ASTA significantly induced the movement speed, displacement, and accumulated distance of MLE-12 cells (Figure 2(B) to (D)). The above results all confirm that ASTA could increase the exercise activity of MLE-12 cells.

ASTA inhibits apoptosis of MLE-12 cells under inflammatory condition

Inflammation leads to cell damage and induces apoptosis.[15] Therefore, we determined the effects of ASTA on the apoptosis of MLE-12 cells under inflammatory condition using flow cytometry. Our results showed that ASTA decreased MLE-12 cell apoptosis under inflammatory condition (Figure 3(A)).

We have confirmed in the above experiments that ASTA can maintain the proliferation and motility of MLE-12 cells under inflammatory environment. Generally, the caspase pathway is a key pathway for cell death induced by inflammation. We detected the changes of caspase 3 and caspase 9 in MLE-12 cell. As in Figure 3(B), caspase 3 and caspase 9 levels were decreased by ASTA. These data reveal that ASTA prevents apoptosis stimulated by LPS in MLE-12 cells.

ASTA inhibits NF-κB pathway activation

As a classic inflammatory signaling pathway,[16] NF-κB pathway is closely related to many diseases caused by inflammation.[17] In the above experimental results, we found that ASTA and NF-κB-targeted small molecule PDTC have inhibitory effects on apoptosis. On one hand, it shows that the targeted blocking of NF-κB signaling pathway has protective effect on inflammatory injury of MLE-12 cells.

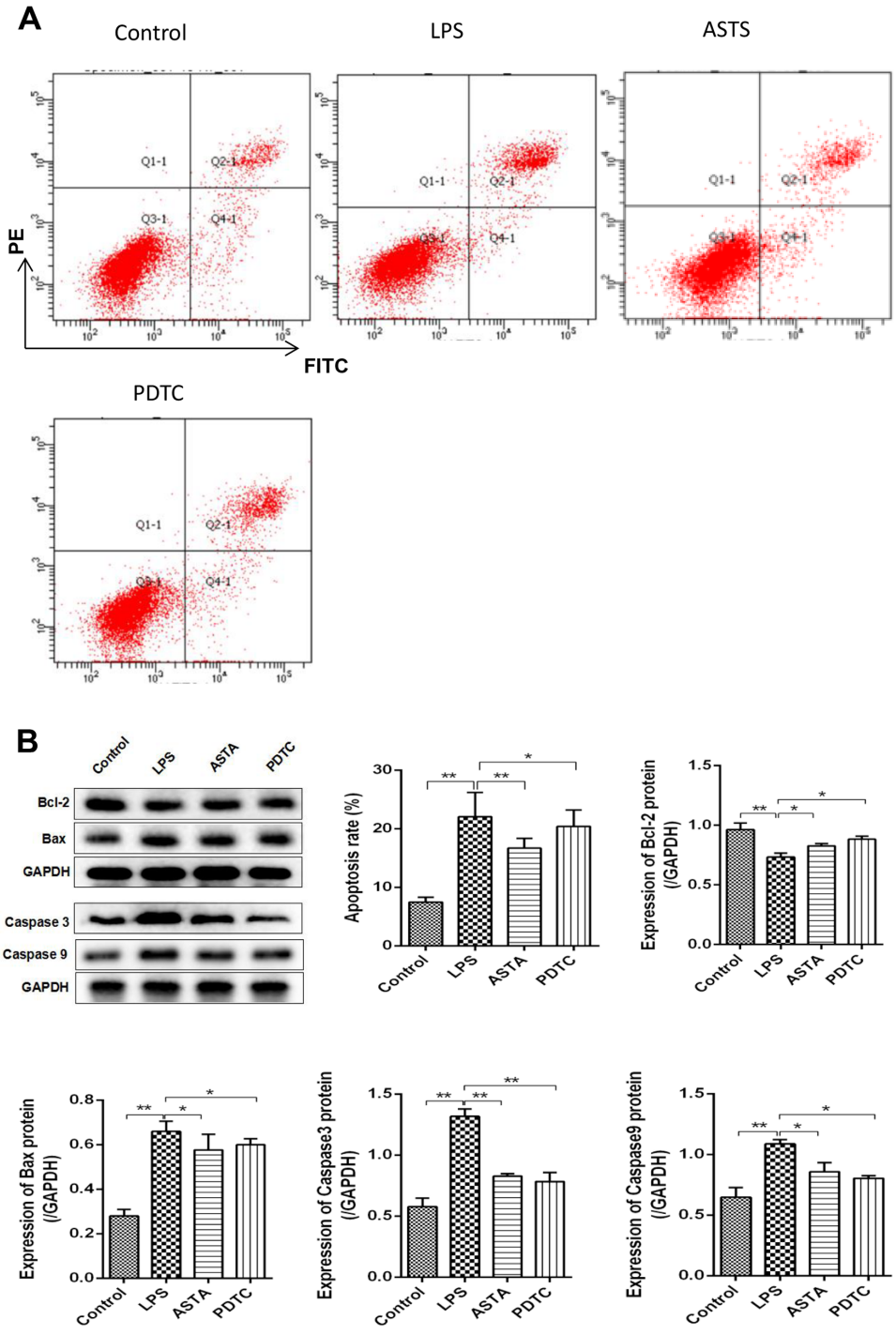


Figure 3. ASTA attenuates LPS-mediated apoptosis in MLE-12 cells. (A) Flow cytometry analyzed apoptosis after different treatments. (B) Western blotting showing Bax, Bcl-2, caspase 9, and caspase 3 expressions in MLE-12 cells. Data are described with as mean value ± SD.
Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$.

On the other hand, it suggests that in MLE-12 cells, ASTA may play a protective effect on inflammatory injury through the NF-κB pathway.

To confirm our inference, the expression of key proteins of the NF-κB pathway was evaluated by the western blot. As in Figure 4(A), the key proteins of the NF-κB pathway were significantly downregulated by ASTA. Importantly, ASTA suppressed the nuclear translocation of NF-κB (Figure 4(B)). Therefore, we determined that ASTA can inhibit the NF-κB pathway.

ASTA reduces inflammatory damage to the lungs

Because ASTA and PDTC have a more obvious effect on promoting proliferation and inhibiting apoptosis of MLE-12 cells under inflammatory condition. We further observed

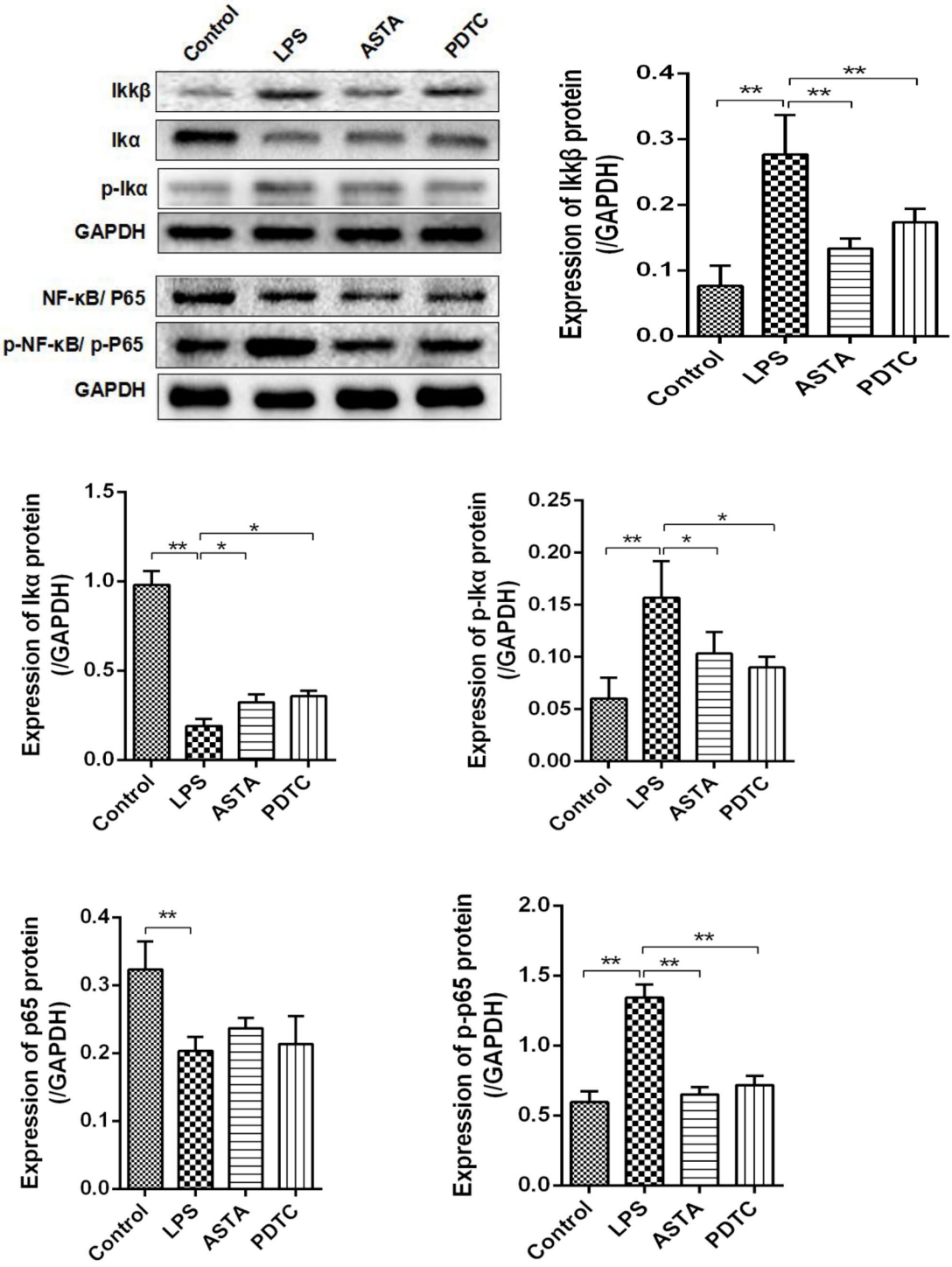
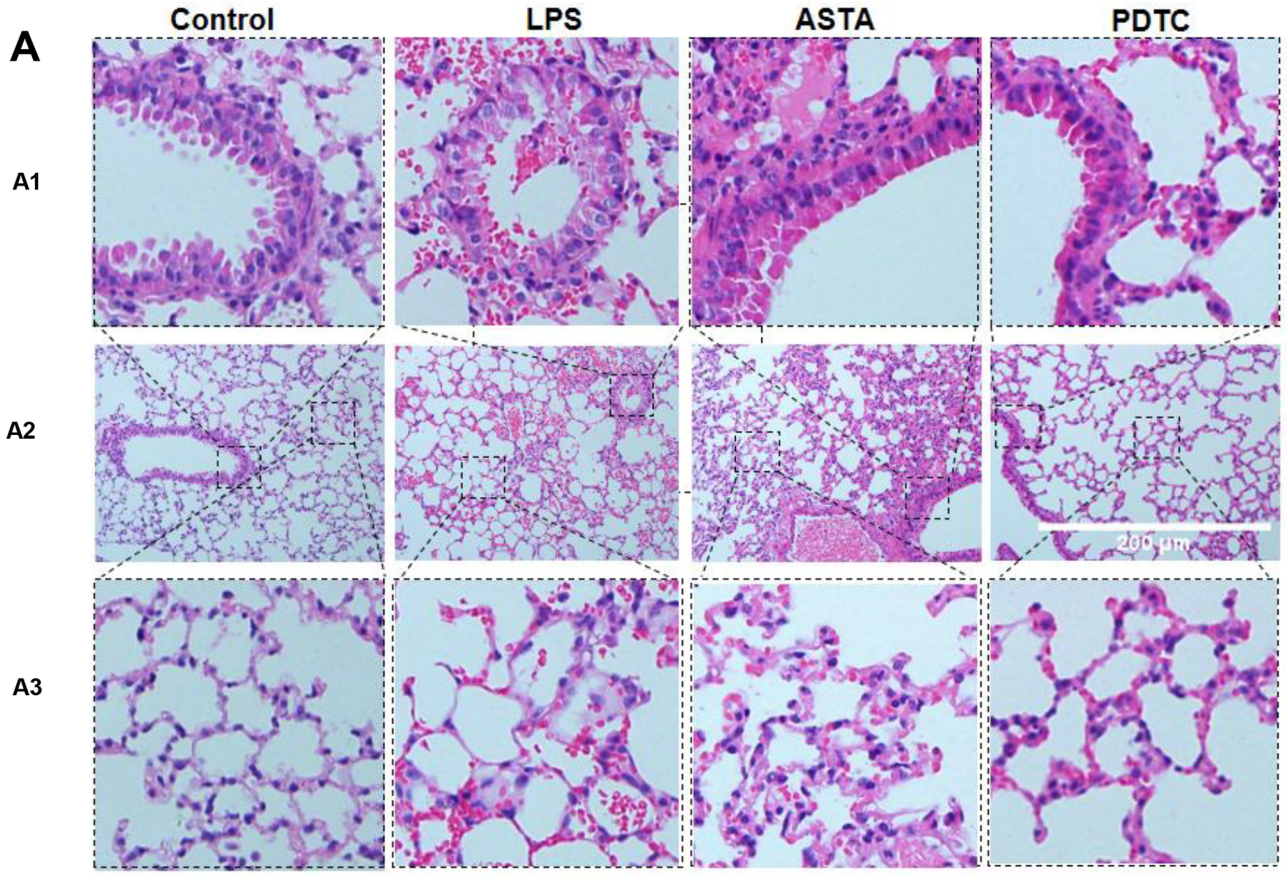


Figure 4. ASTA inhibits apoptosis by targeting NF-κB signal pathway in MLE-12 cells. Western blotting showing the protein expression of Ikkβ, Ikα, p-Ikα, p65, and p-p65 in MLE-12 cells. Data are described with mean value ± SD.
Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$.

the pharmacodynamic effect of ASTA on the inflammatory lung injury model established by LPS in mice. As shown in Figure 5(A), H&E staining of lungs showed that after LPS treatment, the edema, alveolar and bronchial epithelium inflammation injury and hemorrhage were aggravated. These pathological changes were ameliorated markedly by ASTA. Inflammatory mediators are the key substances that induce inflammatory damage, so we further detected IL-6, TNF-α, and NO levels, which are important inflammatory mediators that promote inflammation in the lungs, as well as the levels of IL-10, a cytokine that inhibits inflammation. As shown in Figure 5(B), IL-6, TNF-α, and NO levels in the lungs of mice in the inflammatory injury model group increased significantly, and the level of IL-10 was decreased. Both ASTA and PDTC can reverse these changes. The above experimental results further demonstrate that NF-κB is a



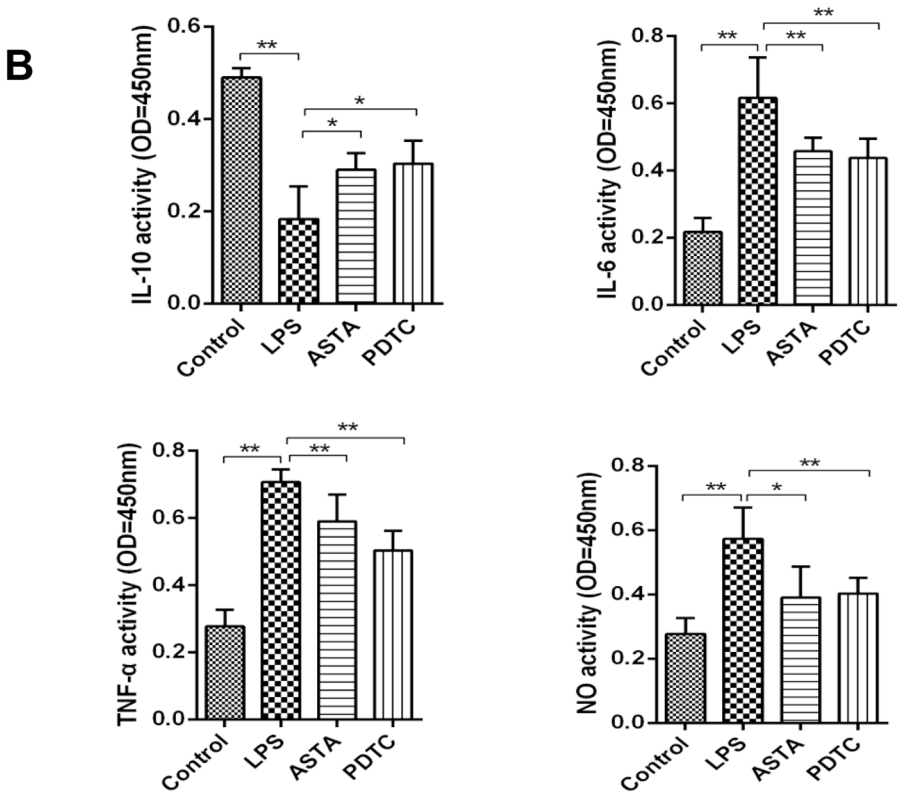


Figure 5. ASTA inhibits apoptosis of lung cancer cells. (A) HE staining of lung tissue (scale bar = 200 μm). Figures A1 and A3 are enlarged images of the square block of Figure A2. (B) The content of IL-10, IL-6, TNF-α, and NO in lung tissue.

key signaling pathway that causes inflammatory damage, and ASTA has the effect of protecting lung tissue from inflammatory damage.

Discussion

How to prevent PTB-related inflammatory pulmonary injury is a problem that this research hopes to solve. Herein, we first used LPS to establish an *in vitro* inflammatory injury model of lung epithelial cells. We found that LPS resulted in inhibition of proliferation and decreased exercise capacity in MLE-12 cells. But we cannot sure that the reduction in cell numbers is simply due to inhibition of proliferation. Inflammatory injury usually also triggers Caspase signaling, leading to apoptosis.[18] Therefore, we found that LPS could significantly promote MLE-12 cell apoptosis.

NF-κB affects cytokine production, cell survival, and cell apoptosis. It takes part in the immune and inflammatory responses. There is a close relationship between the inflammatory pulmonary injury and the abnormal activation of NF-κB pathway.[19,20] We also experimentally found that LPS activates the NF-κB pathway. We treated with the NF-κB inhibitor PDTC, apoptosis was significantly alleviated. Based on the above analysis, we believe that inflammation can induce lung epithelial cell apoptosis through the NF-κB signaling pathway, thereby causing lung tissue damage.

Anti-inflammatory effects of ASTA have been proven. Therefore, we hope to explore whether ASTA can protect lung epithelial cells under inflammatory condition through cell experiments. Our results showed that ASTA maintained MLE-12 cell proliferation and motility under inflammatory condition. More importantly, ASTA reversed LPS-stimulated apoptosis in MLE-12 cells. Based on this, we confirmed that ASTA is able to protect MLE-12 cells from inflammatory damage. We found that both ASTA and PDTC have good antiapoptotic effects, so we further observed the effect of ASTA on NF-κB signaling pathway. The experimental results suggest that ASTA can significantly suppress the excessive activation of the NF-κB pathway. These studies demonstrate that ASTA can suppress the NF-κB signaling pathway, thus reducing apoptosis stimulated by LPS in MLE-12 cells. Meanwhile, it is suggested that ASTA has the potential to protect against pulmonary inflammatory damage.

We further verified the protective function of ASTA against inflammatory lung injury through *in vivo* experiments. HE staining showed that ASTA significantly reduced LPS-induced alveolar and organ damage. Inflammatory mediator production in lung tissue was also significantly reduced. Therefore, we further confirmed that ASTA can reduce lung inflammation and protect lung tissue from inflammatory damage. We believe that ASTA is a natural compound with great potential for protection against inflammatory damage to lung tissue. ASTA may be considered for further development as a drug to prevent inflammatory damage during the treatment of pulmonary TB.

Conclusions

NF-κB is a key signaling pathway that causes inflammatory damage. LPS activates the NF-κB signaling pathway. ASTA can reduce apoptosis stimulated by LPS in MLE-12 cells through suppressing the NF-κB pathway, and can reduce lung inflammation, and protect lung tissue against inflammatory damage.

AUTHORS' CONTRIBUTIONS

AA and LL participated in data analysis. HG, ZA, SY, BY, JR, and AA conducted the experiments. XZ provided reagents. AA and XZ wrote the manuscript. All authors participated in the design and interpretation of the study and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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