Amygdalin Attenuates Airway Epithelium Apoptosis, Inflammation, and Epithelial-Mesenchymal Transition through Restraining the TLR4/NF- κ B Signaling Pathway on LPS-Treated BEAS-2B Bronchial Epithelial Cells

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Keywords

Amygdalin · Cough-variant asthma · Epithelium · Apoptosis · Inflammation · Epithelial-mesenchymal transition · Toll-like receptor 4/NF- κ B signaling pathway

Abstract

Background: Cough-variant asthma (CVA) is a special type of asthma, solely manifesting with coughing. Studies suggest that airway inflammation is associated with CVA pathogenesis. Amygdalin is found to have an anti-inflammatory potential, while how it affects CVA remains unexplored.

Methods: Cytotoxicity delivered by various concentrations of LPS and amygdalin on BEAS-2B cells was determined by Cell Counting Kit-8 assay. CVA in vitro models were established via LPS exposure on BEAS-2B cells which underwent amygdalin pretreatment. Cell apoptosis was determined by flow cytometry. Production of tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, and mucin 5AC (MUC5AC) in BEAS-2B cells was measured by ELISA and qRT-PCR. Expressions of TLR4, E-cadherin, N-cadherin, α -smooth muscle actin (SMA), vimentin, phosphorylated-p65 (p-p65), p65, phosphorylated-I κ B α (p-I κ B α), and I κ B α in BEAS-2B cells were measured by qRT-PCR or Western blot. **Results:** LPS and high concentra-

tions of amygdalin (over 600 μ g/mL) decreased BEAS-2B cell toxicity. Exposure to LPS inhibited toxicity, enhanced apoptosis; and promoted production of TNF- α , IL-6, IL-8, and MUC5AC, increased the levels of N-Cadherin, α -SMA, vimentin, p-p65, and p-I κ B α , and decreased the levels of E-cadherin and I κ B α in BEAS-2B cells. Amygdalin pretreatment counteracted the effects of LPS on BEAS-2B cells. Overexpressing TLR4 reversed amygdalin-exerted effects in LPS-exposed BEAS-2B cells. **Conclusion:** Amygdalin attenuated airway epithelium apoptosis, inflammation and epithelial-mesenchymal transition through restraining the TLR4/NF- κ B signaling pathway in CVA.

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Introduction

Cough-variant asthma (CVA) is a special type of asthma and a frequent disease in children. The only or the main clinical manifestation of CVA arrives at coughing which is characterized by irritant cough in the morning

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or during the night [1]. A lack of timely and effective treatment may prompt some CVA to develop into childhood asthma [2]. Currently, the pathogenesis of CVA is not entirely clear. Nevertheless, existing theories indicate that regional bronchoconstriction, increased cough receptors' sensitivity, inflammation, and heightened vagal activity are contributing factors for CVA [3]. Cough reflex in CVA is complex and it involves the actions of peripheral receptors which are located in epithelial surface and can be stimulated by serotonin, bradykinin, nicotine, protons, capsaicin, ozone, and adenosine [3]. Exogenous deleterious factors like these may further evoke inflammatory responses in airway epithelium that functions as the primary defender of lung [4].

Airway inflammation can lead to damage and dysfunction of airway epithelial cells, which thereby aggravates the pathogenesis and progression of respiratory diseases [5]. Numerous studies designed for discovering drugs that alleviates airway inflammation demonstrated epithelial cell apoptosis and release of inflammatory factors in airway disease models [6]. Furthermore, long duration of inflammation in airways is correlated to a greater degree of airway remodeling [7, 8]. Progressive remodeling can cause irreversible airway epithelial dysfunction [8]. Airway epithelial dysfunction resulting from damaged airway epithelial cells exhibited reduced epithelial cell adhesion with decreased epithelial markers and increased interstitial markers, hinting that epithelial-mesenchymal transition (EMT) is an important mechanism in airway remodeling [9]. Also, recent studies highlighted that EMT, a cell remodeling process where epithelial cells lose epithelial phenotypes, plays key roles in the progression of pulmonary diseases [10].

Recently, increasing attention has been drawn to the pharmaceutical potentials of amygdalin. Amygdalin, also known as interchangeably vitamin B17 or laetrile, is a cyano-containing glycoside compound derived from seeds of the Rosaceae family [11]. Studies on the pharmaceutical effects of amygdalin have shown that aside from the properties of cancer cell growth blocking and anti-atherosclerosis [12, 13], the prevalent activities exerted by amygdalin are inflammation relieving [14], cough suppression, and phlegm elimination [15]. However, how it affects CVA remains unexplored.

Moreover, emerging studies have revealed that manipulation on TLR4/NF- κ B signaling ameliorated LPS-induced bronchial epithelial cell injury and inflammation [16]. However, the approaches to manipulating TLR4/NF- κ B signaling pathway for the objective of airway inflammation relieving remain insufficiently investigated.

In this research, we explored the mechanism by which amygdalin exerts anti-inflammatory effects and how the activation of the TLR4/NF- κ B signaling pathway affects this mechanism in CVA. As LPS is applied to simulate asthmatic conditions in vitro [17], this study was conducted on bronchial epithelial cells under LPS-mimicked asthmatic condition.

Materials and Methods

Cell Culture

Human bronchial epithelial cells (BEAS-2B cells, CRL-9609) [16] were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were housed in an atmosphere of 37°C and 5% CO₂ and cultured using BEGM Bronchial Epithelial Cell Growth Medium BulletKits (Lonza/Clonetics, Alpharetta, GA, USA).

Drug Treatment

Amygdalin (A6005, C₂₀H₂₇NO₁₁, purity: ≥99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, D2650, Sigma-Aldrich, St. Louis, MO, USA). For preparation of working solutions used in cell pretreatment, amygdalin was diluted into solutions with various concentrations of 100, 200, 400, 600, and 1,000 µg/mL. BEAS-2B cells were treated with the amygdalin working solutions for 24 h at 37°C with 5% CO₂, prior to CVA model establishment.

LPS Exposure

LPS (L2630, Sigma-Aldrich, St. Louis, MO, USA) were used for establish in vitro CVA models following amygdalin pretreatment. When BEAS-2B cells grew to 80% confluence, they were exposed to LPS at various concentrations (1, 5, 10, 25, and 50 µg/mL) or a specific concentration (25 µg/mL) for CVA model establishment for 24 h at 37°C with 5% CO₂.

Cell Transfection

TLR4 overexpression plasmid constructed using pcDNA3.1 vector was purchased from ThermoFisher (Waltham, MA, USA). Before amygdalin pretreatment and LPS exposure, BEAS-2B cells were transfected with TLR4 overexpression plasmid using Lipofectamine 3000 transfection reagent (L3000015, ThermoFisher, Waltham, MA, USA). Briefly, BEAS-2B cells were plated to be 3 × 10⁴ cells/well in 96-well plates. Until the cell confluence reached 90% confluence, Lipofectamine 3000 transfection reagent and TLR4 overexpression plasmid were diluted by Opti-MEM medium (31985062, ThermoFisher, Waltham, MA, USA). All the diluted genes were first incubated with the diluted Lipofectamine 3000 transfection reagent at 37°C for 20 min. The incubated solution was added into the cells, followed by the incubation with gene-lipid complex for 48 h at 37°C.

Cell Counting Kit-8 Assay

The cytotoxicity induced by LPS and amygdalin on BEAS-2B cells was determined using Cell Counting Kit (CCK)-8 reagent (20140419, Beyotime, Beijing, China). BEAS-2B cells were seeded at 2 × 10³ cells/well in 96-well plates. After TLR4 transfection,

Table 1. Primers used in qRT-PCR for the target genes

Gene	Species	Forward	Reverse
TNF- α	Human	5'-CCTCTCTCTAATCAGCCCTCTG-3'	5'-GAGGACCTGGGAGTAGATGAG-3'
IL-6	Human	5'-ACTCACCTCTTCAGAACGAATTG-3'	5'-CCATCTTTGGAAGGTTTCAGGTTG-3'
IL-8	Human	5'-TTTGTGCAAGGAGTGCTAAAGA-3'	5'-AACCTCTGCACCCAGTTTTTC-3'
MUC5AC	Human	5'-TGCCCTACAACAAGAACAAC-3'	5'-GGAACAGCACTGGGAGTAGTT-3'
TLR4	Human	5'-AGACCTGTCCCTGAACCCCTAT-3'	5'-CGATGGACTTCTAAACCAGCCA-3'
E-cadherin	Human	5'-CGAGAGCTACACGTTACCGG-3'	5'-GGGTGTCGAGGGAAAAATAGG-3'
N-cadherin	Human	5'-AGCCAACCTTAAGTGGAGAGT-3'	5'-GGCAAGTTGATTGGAGGGATG-3'
α -SMA	Human	5'-AAAAGACAGCTACGTGGGTGA-3'	5'-GCCATGTTCTATCGGGTACTTC-3'
Vimentin	Human	5'-AGTCCACTGAGTACCGGAGAC-3'	5'-CATTTACGCATCTGGCGTTC-3'
GAPDH	Human	5'-GAGAAGGCTGGGGCTCATTT-3'	5'-AGTGATGGCATGGACTGTGG-3'

TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IL-8, interleukin-8; MUC5AC, mucin 5AC; α -SMA, α -smooth muscle actin.

amygdalin pretreatment, and LPS exposure, CCK-8 reagent (10 μ L) was added into the seeded cells in each well, followed by incubation for 2 h at 37°C with 5% CO₂. Cell absorbance at 450 nm used as the indicator of cell toxicity was recorded by a microplate spectrophotometer (Epoch 2, BioTek, Winooski, VT, USA).

Annexin V/Propidium Iodide Staining Assay

After TLR4 transfection, amygdalin pretreatment, and LPS exposure, BEAS-2B cell apoptosis was determined using annexin V-FITC/propidium iodide (PI) apoptosis detection kits (E-CK-A211, Elabscience, Shanghai, China, <http://www.yeasen.com/products/detail/944>). Briefly, after being digested using Trypsin (PP0100, Sigma-Aldrich, St. Louis, MO, USA), BEAS-2B cells were centrifuged, washed by PBS, and last resuspended by binding buffer. Annexin V-FITC solution (5 μ L) and PI solution (10 μ L) were added for reaction in the cells for 20 min at room temperature in the darkness. Later, the cells were subjected to flow cytometer (Cytometer, Beckman Coulter, Brea, CA, USA) and analyzed by CytExpert software (version 2.2.0.97, Beckman Coulter, Brea, CA, USA).

Enzyme-Linked Immunosorbent Assay

After TLR4 transfection, amygdalin pretreatment and LPS exposure, the production of tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-8 in BEAS-2B cells were measured by ELISA kits (KGEHC103 α , KGEHC007, KGEHC008, Keygentec, Nanjing, China). Briefly, BEAS-2B cells was centrifuged at 2,000 g for 20 min for supernatant collection. The supernatant was added into enzyme-label coated plates, followed by incubation at 36°C for 90 min. Then, the biotinylated antibody was added into the plates, again followed by incubation at 36°C for 60 min. Later, the enzyme-conjugated working solution were added into the plates, followed by incubation at 36°C for 30 min. Last, the plates were incubated with color-developing reagent at 37°C for 15 min. The reaction was terminated using 2 mol/L H₂SO₄. Optical density at 450 nm, used as the indicator of production level, was measured under a microplate reader (ELx808, BioTek, Winooski, VT, USA).

Quantitative Reverse Transcription Polymerase Chain

Total RNA from BEAS-2B cells were isolated using Trizol reagent (15596026, ThermoFisher, Waltham, MA, USA). Chlo-

roform (151858, Sigma-Aldrich, St. Louis, MO, USA) was used to stratify the RNA, which were then precipitated by isopropanol (19516, Sigma-Aldrich), washed by 75% ethanol (E7023, Sigma-Aldrich), and resuspended in RNase-free water (10977023, ThermoFisher). cDNA was synthesized using SuperScript IV reverse transcriptase (18090010, ThermoFisher) and amplified under a PCR detection System (CFX Connect, Bio-Rad, Philadelphia, PA, USA) using PowerUp SYBR Green Master Mix (A25742, ThermoFisher). The primers used were listed in Table 1. Real-time PCR reaction was initiated at 95°C predenaturation for 10 min, 95°C denaturation for 15 s and 40 circles of 95°C annealing for 15 s, and 60°C elongation for 60 s. The relative gene expressions were estimated by 2^{- $\Delta\Delta$ Ct} method [18].

Western Blot

Total protein lysates from BEAS-2B cells were obtained using RIPA Buffer (89900, ThermoFisher). The protein lysates were quantitated using BCA kits (A53227, ThermoFisher). Thirty-five μ g of the protein lysates and 4 μ L of marker (PR1910, Solarbio, Beijing, China) were separately loaded, electrophoresed by 8% SDS-PAGE gel (P0051A, Beyotime, Shanghai, China). The electrophoresed protein lysates were transferred onto PVDF membranes (P2438, Sigma-Aldrich) and blocked using 5% skim milk in Tris-Buffered Saline with 1% Tween 20 (TBST, TA-125-TT, ThermoFisher) for 1 h at room temperature. Then, the membranes underwent overnight incubation at 4°C with primary antibodies for E-cadherin (ab40772, 97 kDa, 1:10,000, Abcam, Cambridge, MA, USA), N-cadherin (ab18203, 130 kDa, 1:1,000), α -smooth muscle actin (SMA) (ab5694, 42 kDa, 1:1,000), vimentin (ab92547, 54 kDa, 1:1,000), TLR4 (ab13867, 90 kDa, 1:500), phosphorylated-p65 (p-p65) (ab86299, 60 kDa, 1:2,000), p65 (ab16502, 64 kDa, 1:1,000, Abcam), phosphorylated-I κ B α (p-I κ B α) (#2859S, 40 kDa, 1:1,000, Cell Signaling, Beverly, MA, USA), I κ B α (#4814S, 39 kDa, 1:1,000, Cell Signaling), and GAPDH (ab8245, 36 kDa, 1:500, Abcam). Following washing with TBST, the membranes were incubated with Goat anti-Rabbit IgG (A32731, 1:10,000, ThermoFisher) or Goat anti-mouse IgG (A32733, 1:1,000, ThermoFisher). The protein bands were visualized using enhanced chemiluminescence reagent kit (WP20005,

Fig. 1. Amygdalin and LPS might decrease BEAS-2B cell toxicity, and amygdalin counteracted an LPS-induced decreasing effect on BEAS-2B cell toxicity. **a** BEAS-2B cell toxicity was determined by CCK-8 assay after exposure to various concentrations of LPS (0, 1, 5, 10, 25, and 50 µg/mL). **b** BEAS-2B cell toxicity was determined by CCK-8 assay after treatment with various concentrations of

amygdalin (0, 50, 100, 200, 400, 600, and 1,000 µg/mL). **c** The toxicity of BEAS-2B cells pretreated with various concentrations of amygdalin (0, 50, 100, 200, 400, 600, and 1,000 µg/mL) was determined by CCK-8 assay after exposure to LPS (25 µg/mL). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *versus control; +versus LPS. CCK-8, Cell Counting Kit-8; MUC5AC, mucin 5AC.

ThermoFisher) and analyzed by ImageJ software (1.52s version, National Institutes of Health, Bethesda, MA, USA).

Statistical Analysis

All data were presented as mean $\pm$ SD. Statistical analysis was performed using GraphPad Prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA). Comparison of differences among multiple groups was subjected to one-way ANOVA, and pairwise comparison of differences was subjected to Tamhane test. $p < 0.05$ was considered statistically significant.

Results

LPS Might Decrease BEAS-2B Cell Toxicity, and Amygdalin Counteracted an LPS-Induced Decreasing Effect on BEAS-2B Cell Toxicity

For selection of an optimum concentration of LPS for establishment of in vitro models of CVA, BEAS-2B cells were exposed to increasing concentrations of LPS (0, 1, 5, 10, 25, and 50 µg/mL). The results of cytotoxicity assay performed using CCK-8 kits showed that exposure to LPS at 5, 10, 25, and 50 µg/mL significantly decreased BEAS-2B cell toxicity, compared to the LPS-unexposed cells ($p < 0.05$, $p < 0.001$, Fig. 1a). Then, the effect of amygdalin on BEAS-2B cell toxicity was examined through treatment with different concentrations of amygdalin (0, 50, 100, 200, 400, 600, and 1,000 µg/mL) on the cells. The results of CCK-8 assay showed that amygdalin treatment at 600 and 1,000 µg/mL decreased BEAS-2B cell toxicity, the other concentrations below 600 µg/mL produced no ob-

vious effects on the toxicity, compared to the amygdalin-untreated cells ($p < 0.01$, $p < 0.001$; Fig. 1b). Subsequently, as LPS at 25 µg/mL ensured a pronounced cytotoxicity on BEAS-2B cells, it was chosen for establishment of in vitro models of CVA. After treatment with amygdalin at 100, 200, and 400 µg/mL, the toxicity of LPS-exposed BEAS-2B cells exhibited a significant increase, compared to the cells without amygdalin treatment ($p < 0.05$, $p < 0.01$, $p < 0.001$; Fig. 1c). All these results suggested a protective role of amygdalin on CVA.

Amygdalin Counteracted LPS-Induced Apoptosis and Inflammatory Responses in BEAS-2B Cells

Next, amygdalin at 200 and 400 µg/mL that produce no obvious cytotoxicity to the BEAS-2B cells was used to treat the cells, for investigation of the effect of amygdalin on apoptosis and inflammatory factor release in CVA. BEAS-2B cell apoptosis was determined using annexin V-FITC/PI staining analysis. The results of flow cytometry showed that BEAS-2B cell apoptosis was enhanced under exposure to LPS ($p < 0.001$), while the BEAS-2B cell apoptosis following exposure to LPS was inhibited by treatment with amygdalin ($p < 0.01$, $p < 0.001$; Fig. 2a, b).

Besides that, inflammation was also a frequent consequence in asthma. Previous studies have maintained that an increased level of MUC5AC, a proverbial mucus protein which contributes to the mucous phenotypes, is critical to inflammatory response induction in asthma [19, 20]. Thus, we deciphered how amygdalin affects the pro-

Fig. 2. Amygdalin counteracted LPS-induced apoptosis and inflammatory responses in BEAS-2B cells. **a, b** The apoptosis of BEAS-2B cells pretreated with amygdalin (200 and 400 µg/mL) was determined by flow cytometry after exposure to LPS (25 µg/mL). **c–f** The levels of TNF-α, IL-6, IL-8, and MUC5AC in BEAS-2B cells pretreated with amygdalin (200 and 400 µg/mL) was measured by ELISA after exposure to LPS (25 µg/mL). **g** The mRNA

expressions of TNF-α, IL-6, IL-8, and MUC5AC in BEAS-2B cells pretreated with amygdalin (200 and 400 µg/mL) were analyzed by qRT-PCR after exposure to LPS (25 µg/mL). # $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *versus Control; #versus LPS. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-8, interleukin-8; MUC5AC, mucin 5AC; α-SMA, α-smooth muscle actin.

duction of inflammatory factors, including TNF-α, IL-6, IL-8, as well as MUC5AC in CVA. The results of ELISA assay and qRT-PCR analyses revealed that the levels and mRNA expressions of TNF-α, IL-6, IL-8, and MUC5AC in BEAS-2B cells were increased after exposure to LPS, while treatment with amygdalin inhibited those levels and expressions in LPS-exposed BEAS-2B cells ($p < 0.01$, $p < 0.001$; Fig. 2c–g).

Amygdalin Counteracted LPS-Induced EMT and Activation of TLR4/NF-Kb Signaling in BEAS-2B Cells

EMT, a cell remodeling process where epithelial cells lose epithelial phenotypes, is associated with the progression of chronic obstructive pulmonary disease [21]. We therefore examined the expressions of EMT-specific markers in CVA. The results of Western blot confirmed

that exposure to LPS led to downregulation of E-cadherin and upregulation of N-cadherin, α -SMA, and vimentin in BEAS-2B cells ($p < 0.001$), while treatment with amygdalin upregulated E-cadherin and downregulated N-cadherin, α -SMA, and vimentin in LPS-exposed BEAS-2B cells ($p < 0.05$, $p < 0.01$; Fig. 3a, b). Moreover, recent studies have demonstrated increased activation of the TLR4/

NF- κ B signaling pathway in asthmatic mice [22]. To determine whether CVA is driven by the activation of TLR4/NF- κ B signaling pathway, the pathway related-markers was examined. The results of Western blot revealed that the levels of TLR4 and p-p65 and p-I κ B α were increased, and I κ B α level and the ratios of p-p65 to p65 and p-I κ B α to I κ B α were decreased in BEAS-2B cells after exposure

Fig. 3. Amygdalin counteracted LPS-induced EMT and activation of TLR4/NF- κ B signaling in BEAS-2B cells. **a, b** The protein expressions of E-cadherin, N-cadherin, α -SMA, and vimentin in BEAS-2B cells pretreated with amygdalin (200 and 400 μ g/mL) after exposure to LPS (25 μ g/mL) were analyzed by Western blot, with GAPDH serving as a reference gene. **c, d** The protein expressions of TLR4, p-p65, p65, p-I κ B α , and I κ B α in BEAS-2B cells pretreated

with amygdalin (200 and 400 μ g/mL) after exposure to LPS (25 μ g/mL) were analyzed by Western blot, with GAPDH serving as a reference gene. **e, f** The ratios of p-p65 to p65 and p-I κ B α to I κ B α in BEAS-2B cells pretreated with amygdalin (200 and 400 μ g/mL) after exposure to LPS (25 μ g/mL) $^{\#}p < 0.05$; $^{**}p$ or $^{***}p < 0.01$; $^{***}p$ or $^{###}p < 0.001$; * versus control; $^{\#}$ versus LPS, p-p65, phosphorylated-p65; p-I κ B α , phosphorylated-I κ B α ; α -SMA, α -smooth muscle actin.

to LPS ($p < 0.001$), while treatment with amygdalin resulted in decreased levels of TLR4 and p-p65 and p-I κ B α , an increased I κ B α level, and reduced ratios of p-p65 to p65 and p-I κ B α to I κ B α in LPS-exposed BEAS-2B cells ($p < 0.05$, $p < 0.01$; $p < 0.001$, Fig. 3a–f). These results suggested that the protective effects of amygdalin on LPS-injury BEAS-2B cells were exerted through inhibiting EMT and the TLR4/NF- κ B signaling pathway.

Overexpressing TLR4 Reversed Amygdalin-Exerted Effects on Toxicity, Apoptosis, and Inflammatory Responses via TLR4/NF- κ B Signaling in LPS-Exposed BEAS-2B Cells

For verification on the notion that the inhibition of the TLR4/NF- κ B signaling pathway is indispensable in the effects exerted by amygdalin against CVA, TLR4 overexpression plasmid was utilized to promote the activation of the TLR4/NF- κ B signaling pathway. Transfection of TLR4 overexpression plasmid led to an increased TLR4 expression, compared to the LPS + amygdalin + NC group ($p < 0.01$), and it reversed the amygdalin-exerted promotive effect on LPS-decreased BEAS-2B cell toxicity and the amygdalin-exerted inhibitory effect on LPS-enhanced apoptosis ($p < 0.001$; Fig. 4a–d). Furthermore, amygdalin-exerted inhibitory effect on LPS-induced production of TNF- α , IL-6, IL-8, and MUC5AC was also reversed by transfection of TLR4 overexpression plasmid ($p < 0.01$, $p < 0.001$; Fig. 4a–d). Simultaneously, transfection of TLR4 overexpression plasmid reversed the amygdalin-exerted inhibitory effect on LPS-upregulated N-cadherin, α -SMA, and vimentin, and it reversed the amygdalin-exerted promotive effect on LPS-downregulated E-cadherin ($p < 0.05$, $p < 0.01$, $p < 0.001$; Fig. 5a, b). Besides, transfection of TLR4 overexpression plasmid increased the levels of p-p65 and p-I κ B α , raised the ratios of p-p65 to p65 and p-I κ B α to I κ B α , and decreased p-I κ B α level during amygdalin treatment on LPS-injured BEAS-2B cells ($p < 0.05$, $p < 0.001$; Fig. 5c, d).

Fig. 4. Overexpressing TLR4 reversed amygdalin-exerted effects on toxicity, apoptosis, and inflammatory responses in LPS-exposed BEAS-2B cells. **a** TLR4 mRNA expression in TLR4 overexpression plasmid-transfected BEAS-2B cells after pretreatment with amygdalin (200 and 400 μ g/mL) and exposure to LPS (25 μ g/mL) was analyzed by qRT-PCR. **b** The toxicity of TLR4 overexpression plasmid-transfected BEAS-2B cells after pretreatment with amygdalin (200 and 400 μ g/mL) and exposure to LPS (25 μ g/mL) was determined by CCK-8 assay. **c, d** The apoptosis of TLR4 overexpression plasmid-transfected BEAS-2B cells after pretreatment with amygdalin (200 and 400 μ g/mL) and exposure to LPS (25 μ g/mL) was determined by flow cytometry. **e–h** The levels of

Discussion

CVA, considered as the precursor of classic asthma, has some clinical profiles that are the same as classic asthma dose, demonstrating eosinophilic inflammation of bronchi and sputum eosinophilia [23]. Existing guidelines for treatment of CVA as well as CA recommend that CVA patients should resort to inhalation of corticosteroids and bronchodilators [24]. However, inhaling high-dose of bronchodilators and corticosteroids can elicit vascular remodeling effects, which may include side effects that thus aggravate symptoms [25]. Therefore, discovering a natural occurring agent that harbors less adverse effects during treatment of CVA is of great importance. In this study, we demonstrated that amygdalin, a traditional herbal medicine, could increase toxicity, inhibit apoptosis, reduce production of inflammatory factors and mucus protein, and inhibit EMT in LPS-injured epithelial cells through inhibition of the TLR4/NF- κ B signaling pathway.

Epithelial cell damage can result from airway inflammation which is a distinct pathological feature of asthma [26]. Accumulating studies have confirmed that exposure to LPS causes airway inflammation and injury [17, 27]. Our study applied various concentrations of LPS on BEAS-2B cells to find out 25 μ g/mL as the optimum concentration of LPS for establishment of CVA models. Recent studies set for finding cures for pulmonary disease have illustrated that treatment with <600 μ g/mL of amygdalin has no effects on BEAS-2B cell toxicity, and the concentration over 600 μ g/mL delivers an inhibitory effect on the viability [28]. Replicating these findings, our study again showed that 400 μ g/mL was the highest concentration of amygdalin, which did not reduce toxicity of BEAS-2B cells. Furthermore, amygdalin is found to counteract cigarette smoke-delivered negative effect on cell viability [28]. Similarly, following the establishment of CVA models, we found that treatment with amygdalin at lower than

TNF- α , IL-6, IL-8, and MUC5AC in TLR4 overexpression plasmid-transfected BEAS-2B cells after pretreatment with amygdalin (200 and 400 μ g/mL) and exposure to LPS (25 μ g/mL) were measured by ELISA. **i** The mRNA expressions of TNF- α , IL-6, IL-8, and MUC5AC in TLR4 overexpression plasmid-transfected BEAS-2B cells after pretreatment with amygdalin (200 and 400 μ g/mL) and exposure to LPS (25 μ g/mL) were analyzed by qRT-PCR. $^{\#}p < 0.05$; $^{\#\#}p$ or $^{\wedge}p < 0.01$; $^{***}p$ or $^{###}p$ or $^{\wedge\wedge\wedge}p < 0.001$; * versus control; $^{\#}$ versus LPS; $^{\wedge}$ versus LPS + amygdalin + NC. TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IL-8, interleukin-8; MUC5AC, mucin 5AC; CCK-8, Cell Counting Kit-8.

study demonstrated an apoptosis-inducing effect of LPS. Notably, we found that after amygdalin treatment LPS-induce apoptosis was repressed.

Recent therapeutic strategies that target airway inflammation have been considered as effective to antagonize inflammatory lung pathologies in asthma [31]. Epithelial cells isolated from asthmatic patients displayed promoted release of IL-8 and TNF- α [32]. Meanwhile, markedly increased existence of sputum in the airways has been found in patients with CVA [33]. MUC5AC is a glycoprotein constituting airway mucus [20]. Mucus obstruction can form nutrient-rich nidi for bacterial infections that can trigger neutrophilic airway inflammation [34]. As reported in Shang's studies, LPS-induced in vitro models exhibited releases of TNF- α , IL-6, and IL-8 and increased production of MUC5AC, while anti-inflammatory treatment reversed these phenomena [16]. In addition, EMT is thought to account for airway hyperresponsiveness and remodeling [35]. In our study, LPS induction also caused releases of these inflammatory factors, increased MUC5AC level, and promoted EMT as reflected by aberrantly expressed EMT-specific markers (E-cadherin, N-cadherin, α -SMA, and vimentin). Amygdalin treatment was found by us to reverse all these LPS-induced effects, which confirmed the anti-inflammatory and EMT property of amygdalin.

Previous studies have shown that inhibiting the NF- κ B signaling pathway is a mechanism by which amygdalin suppresses inflammation in acute lung injury [36]. Shang's study has found that restraining the TLR4/NF- κ B signaling results in suppression on LPS-induced epithelial cell injury and inflammation, which is accompanied by repressed expressions of TLR4 and p-p65 [16]. TLR4, one of the TLRs that recognizes a variety of pathogen-associated molecular patterns [37], is found to be activated by LPS to basically trigger all the responses to LPS [38]. TLR4 receptor can elicit the activation of NF- κ B pathway to thereby unleash inflammatory factors [39]. NF- κ B, a transcription factor which is key to inflammatory responses, controls the final immune response through regulating the expressions of inflammatory cytokines and chemokines [40, 41]. Our study found that amygdalin treatment decreased LPS-induced expressions of TLR4, p-p65, and p-I κ B α and increased LPS-antagonized expression of I κ B α (a NF- κ B inhibitor), suggesting that anti-inflammatory effect of amygdalin can be exerted through inhibiting the TLR4/NF- κ B signaling. Subsequently, for verification on the necessity of the inhibition of the TLR4/NF- κ B signaling pathway in amygdalin-induced anti-inflammatory and EMT activities, we overexpressed TLR4 to activate this pathway. Our result showed that overexpressing TLR4 attenuated the effect of amygdalin.

The validation of amygdalin functioning on CVA using other cell lines, animal models, or the important proteins/pathways in patients would be conducted in future, which might be limitations of this research.

In conclusion, amygdalin increased epithelial cell viability by inhibiting the activation of NF- κ B through TLR4. Thereby, amygdalin also reduced apoptosis, EMT, and the synthesis of proinflammatory cytokines. The research might put forward an effective therapeutic agent for airway injury in CVA.

Statement of Ethics

No humans or animals were involved in this research.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

Substantial contributions to conception and design: Z.S. Data acquisition, data analysis and interpretation: B.Z. Drafting the manuscript or critically revising it for important intellectual content: all authors. Final approval of the version to be published: all authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: all authors.

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