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Costunolide ameliorates lipoteichoic acid-induced acute lung injury via attenuating MAPK signaling pathway

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ABSTRACT

Lipoteichoic acid (LTA)-induced acute lung injury (ALI) is an experimental model for mimicking Gram-positive bacteria-induced pneumonia that is a refractory disease with lack of effective medicines. Here, we reported that costunolide, a sesquiterpene lactone, ameliorated LTA-induced ALI. Costunolide treatment reduced LTA-induced neutrophil lung infiltration, cytokine and chemokine production (TNF-α, IL-6 and KC), and pulmonary edema. In response to LTA challenge, treatment with costunolide resulted less iNOS expression and produced less inflammatory cytokines in bone marrow derived macrophages (BMDMs). Pretreatment with costunolide also attenuated the LTA-induced the phosphorylation of p38 MAPK and ERK in BMDMs. Furthermore, costunolide treatment reduced the phosphorylation of TAK1 and inhibited the interaction of TAK1 with Tab1. In conclusion, we have demonstrated that costunolide protects against LTA-induced ALI via inhibiting TAK1-mediated MAPK signaling pathway, and our studies suggest that costunolide is a promising agent for treatment of Gram-positive bacteria-mediated pneumonia.

1. Introduction

Infection of Gram-positive bacterial, such as pathogenic *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae* (*S. pneumoniae*), leads to acute lung injury, which is a worldwide leading cause of mortality [1]. Bacteria pathogenic components not only directly cause tissue injury but also trigger severe inflammatory response that could lead to multiple tissue injury [2]. Lipoteichoic acid (LTA) is one of major pathogenic components derived from Gram-positive bacterial outer cell wall and has been identified to induce inflammatory response and lead to tissue injury [3]. Intratracheal injection of LTA leads to acute pneumonia, which is characterized by lung infiltration of inflammatory cells, alveolar macrophage activation, production of cytokines and chemokines, generation of reactive oxygen and nitrogen species, and pulmonary edema [4, 5]. Therefore, LTA-induced acute lung injury (ALI) not only mimics Gram-positive bacteria-mediated pneumonia but also is an appropriate model for evaluating anti-inflammatory effects of compounds.

Innate immune system protects against pathogenic bacteria infection and maintains homeostasis after infection through pathogen recognition receptors (PRRs) binding and recognizing pathogen-associated molecular patterns (PAMPs) [6, 7]. Toll-like receptor 2 (TLR2), one of PRRs, is well known to be activated by LTA, activates TIR domain-containing adaptor molecules including MyD88, TRIF, TRAM and SARM [8]. Recruitments of these adaptor molecules to TLR2 initiate different active signaling pathways including ubiquitination of TRAF6 and phosphorylation of TAK1, which in turn activate MAPK (p38 MAPK, ERK and JNK) signal pathways and NF-κB signal pathway [9]. These active signal pathways induce transcriptional factor activation and then contribute to activation of macrophages, epithelial cells and endothelial cells, production of cytokines and chemokines that in turn trigger cytokine storm and lead to sepsis in vivo [10]. Therefore, blockage of pro-inflammatory cytokine production (TNF-α and IL-6) or inhibition of TLR2-mediated signaling pathways (NF-κB and MAPK) may be effective therapeutic strategies for treating Gram-positive bacteria such as *S. aureus*-induced ALI [11].

Costunolide, a sesquiterpene lactone, extracted from the Compositae and the Magnoliaceae, has multiple pharmacological activities, including inhibition of tumor cell proliferation, protection from viral and fungal infections, as well as reduction of inflammatory

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response [12–14]. Costunolide has been reported to inhibit several signal inflammatory pathways including NF-κB activation and MAPK activation in RAW 264.7 cells, and STAT3 activation in THP-1 cells [15, 16]. These results suggest that costunolide may target multiple molecules in inflammatory cells.

Although costunolide has been shown to have anti-inflammatory activities, it has not been shown whether costunolide modulates Gam-positive bacteria-induced inflammation and regulates LTA-TLR2-induced signaling pathways. Using LTA-induced murine ALI model, we found that costunolide ameliorated neutrophil lung infiltration, cytokine production, and lung edema. Furthermore, in bone marrow derived macrophages (BMDMs), costunolide treatment attenuated the LTA stimulated production of TNF-α, IL-6 and KC. Meanwhile, costunolide also inhibited the interaction between TAK1 and Tab1, reduced the phosphorylation of TAK1, and attenuated p38 MAPK and ERK activation. Our results suggested that costunolide is a promising agent for treating pneumonia.

2. Materials and methods

2.1. Mice

C57BL/6 male mice were purchased from SLAC Laboratory Animal Corporation (Shanghai, China). Male mice at 8–10 weeks old and weighing 18–22 g housed in specific pathogen-free conditions at the Laboratory Animal Center for this study. All experiments involving mice were in accordance with the Animal Care and Use Committee. This research adhered to the Principles of Laboratory Animal Care (NIH publication No. 85–23, revised 1996).

2.2. LTA-induced acute lung injury

To investigate the effect of costunolide on acute lung injury induced by LTA, we dissolved costunolide in vehicle (10% DMSO, 60% cremophor, 20% ethanol and 10% PBS) for in vivo experiments. Mice were randomly divided into 3 groups (n = 5/group): PBS group, LTA group (LTA + vehicle) and costunolide treated group (LTA + Cos). Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), and intratracheally (i.t.) injected with LTA (100 μg in 50 μL PBS) for 6 h to induce acute lung injury. For costunolide-treated group, mice were pretreated with costunolide (20 mg/kg) for 1 h prior to LTA challenge. At the indicated time, mice were anesthetized and samples (bronchalveolar lavage fluid and lung tissues) were collected.

2.3. Acquisition and analysis of bronchoalveolar lavage fluid (BALF)

The lungs were lavaged three times with 0.6 mL of isotonic PBS (pH 7.4) via a tracheal catheter and BAL fluid was collected and centrifuged at 4 °C, 300 × g for 5 min. The cell-free supernatants were collected for detecting protein concentration using the BCA protein assay kit (Beyotime, Shanghai, China). The cell pellet was resuspended with PBS and counted. Cells were stained with FITC-conjugated anti-mouse Gr-1 (Ly-6G) (BioLegend, USA) for identification of neutrophils, and FITC-conjugated anti-mouse F4/80 and APC-conjugated anti-mouse CD11c (BioLegend, USA) for identification of alveolar macrophages. FACSCanto flow cytometer (Becton Dickinson, USA) and FlowJo 7.6 software were used for analysis.

2.4. Lung histology

Lung tissues were inflated with 4% paraformaldehyde, then tissues were embedded in paraffin and cut into 5-μm-thick sections in a microtome (RM2235, Leica Biosystems, Wetzlar, Germany). Sections were stained with hematoxylin and eosin (H&E) to assess inflammatory cell infiltration. The pulmonary inflammatory injury was quantitated based on the H&E staining slides [17]. Briefly, according to inflammatory cell infiltration, lung edema, hemorrhage, atelectasis and hyaline membrane, the area of pulmonary injury was scored as below: no injury, 0; ~25% of injury, 1; ~50% of injury, 2; ~75% of injury, 3; ~100% of injury, 4.

2.5. MPO activity assay

Lung tissues were homogenized and subjected to three freeze-thaw cycles. After the samples were centrifuged, the supernatants were collected at 4 °C. Adding tetramethylbenzidine into the supernatant, the change of absorbance at 655 nm was measured by the microplate reader (FlexStation 3, Molecular Devices, California, CA, USA). Protein concentration of the supernatants was determined as described above. The MPO activity was defined as the absorbance changes per min per gram protein.

2.6. Lung wet-to-dry weight ratio

Lung tissues were excised at 6 h and rinsed with PBS, then weighed immediately (wet weight), and were heated at 80 °C for 48 h to obtain the dry weight. The wet to dry weight ratios were calculated to evaluate the lung edema.

2.7. RNA isolation, reverse transcription and quantitative PCR

Total RNA was extracted from lung tissues (one of tight upper lobes) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA was reverse-transcribed into cDNA using reverse transcriptase (Toyobo, Osaka, Japan) and amplified by Real-time PCR on StepOne Plus (Thermo Fisher Scientific, Waltham, MA, USA) with primer sets for TNF-α (forward: TTCTCATTCCTGCTTGTGG; reverse: ACTTGGTGGTT TGCTACG), IL-6 (forward: CCACCAAGAACGATAGTCAA; reverse: TTT CCACGATTTCCCAGA), KC (forward: ACCCGCTCGCTTCTCTGT; reverse: AAGGGAGCTTCAGGGTCAAG).

2.8. Cell culture

Femoral and tibia bone marrow was isolated from C57BL/6 wild-type mice. Mouse bone marrow cells were flushed from femurs and tibias with Ca^{2+}/Mg^{2+}-free PBS, then the cellular material was aspirated and centrifuged at 400 × g at 4 °C for 5 min. Cells were then resuspended and incubated with DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 1% penicillin/streptomycin, and 10% L929 cell-conditioned medium for 7 d. Cells were lifted, washed, counted, and replated before study. The cells were treated with various concentration of costunolide (3, 10, 30 μM) according to experiment requirements, 1‰ DMSO was added into culture medium as control. BMDMs were also stimulated with LTA (10 μg/mL) for 6 h after 30 min with costunolide administration. Culture supernatants and cells were collected for RNA, cytokines and protein expression analysis. All cell cultures were maintained at 37 °C with 5% CO_2.

2.9. Measurement of proinflammatory cytokines and chemokine

The concentration of TNF-α, IL-6 and KC in the supernatant from BALF or BMDMs was detected using mouse Quantikine ELISA Kit (R&D SYSTEMS, USA) according to manufactory protocol. The concentrations were calculated based on the standard curves of recombinant TNF-α, IL-6 and KC.

2.10. Western blotting

BMDMs were plated in six-well plates at 2×10^6 cells per well overnight and challenged with LTA (10 μg/mL) after 30 min with costunolide administration. Cells were then collected and lysed in loading buffer. Then the cell lysate were evaluated for protein expression using