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α-Iso-cubebene, a natural compound isolated from *Schisandra chinensis* fruit, has therapeutic benefit against polymicrobial sepsis ☆

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ABSTRACT

α-Iso-cubebene, a natural compound isolated from *Schisandra chinensis* fruit, strongly enhanced survival rate in cecal ligation and puncture (CLP) challenge-induced sepsis. The mechanism involved the marked reduction of viable bacteria in the peritoneal fluid, by virtue of increased phagocytic activity and production of hydrogen peroxide. α-Iso-cubebene also significantly attenuated lung inflammation and widespread immune cell apoptosis in a mouse CLP sepsis model, and inhibited the production of proinflammatory cytokines including tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in CLP mice and lipopolysaccharide-stimulated splenocytes. The results indicate that α-iso-cubebene can reverse the progression of toxic shock by triggering multiple protective downstream signaling pathways to enhance microbial killing and maintain organ function and leukocyte survival.

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1. Introduction

Sepsis is a systemic inflammatory response caused by viable bacteria or bacterial products such as lipopolysaccharide (LPS) [1]. More than 750,000 patients develop sepsis annually in the United States, and the incidence rate is gradually increasing by approximately 1.5% per year [2]. Despite more than 20 years of dedicated research, sepsis remains the major cause of death in intensive care units, and the overall mortality associated with sepsis ranges from 30 to 70% [2,3]. Sepsis-induced lethality is accompanied by failure of proper immune response against invading pathogens [4–6]. Especially, an inability of the innate immune system during early sepsis (i.e., the first 6 h) results in increased mortality. Excessive lymphocyte apoptosis also occurs during sepsis, resulting in clinical signs of multi-organ failure [7,8]. Moreover the levels of the proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1β are substantially increased during sepsis [9–11]. Based on these facts, it is reasonable to hypothesize that the effective treatment of sepsis requires an approach that enhances bactericidal activity, prevents production of inflammatory cytokines, and blocks widespread lymphocyte apoptosis.

In a previous study, we isolated a novel natural compound, α-iso-cubebene, from *Schisandra chinensis* and documented the ability of the compound to stimulate intracellular calcium and increase CXCL8 production in human neutrophils [12]. Subsequently α-iso-cubebene has been reported to inhibit endothelial cell adhesion on monocytes by inhibiting TNF-α-induced reactive oxygen species production and nuclear factor-kappa B (NF-κB) activation [13].

Keeping in mind that endothelial cell adhesion on monocytes and NF-κB activity are important for the induction of important inflammatory mediators including inflammatory cytokines, we presently investigated the in vivo efficacy of α-iso-cubebene in a preclinical mouse model of sepsis, with the aim of defining the mechanisms of septic protection by this novel natural compound.

2. Materials and methods

2.1. Purification of α-iso-cubebene

α-Iso-cubebene was purified from dried fruits of *S. chinensis* as described previously [12]. Briefly, *S. chinensis* (2.5 kg) fruit was ground to a fine powder and was successively extracted at room

Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; CLP, cecal ligation and puncture; ELISA, enzyme-linked immunosorbent assay.

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temperature with *n*-hexane, chloroform ($CHCl_3$), and methanol (MeOH). The hexane extract (308 g) was evaporated in vacuo and chromatographed on a 40 μm, silica gel (J.T. Baker, Phillipsburg, NJ, USA) column (100 × 10 cm) with a step gradient of 0%, 5%, and 20% ethyl acetate in hexane and 5% methanol MeOH in $CHCl_3$ to obtain 38 fractions. Fraction 1 (KH1PA, 3,689 mg) was separated on a silica gel column (100 × 3.0 cm) with 15% acetone in dichloromethane (CH_2Cl_2) to obtain nine fractions. Fraction 2 (KH1PAIB, 999 mg) was separated on a silica gel column (100 × 3.0 cm) with 15% acetone in CH_2Cl_2 to yield α-iso-cubebene (316 mg). Pure α-iso-cubebene (purity >99%) was identified by high-performance liquid chromatography on a Phenomenex Luna C18 column (150 × 4.6 mm internal diameter; 5 μm particle size) with an acetonitrile–water-reagent alcohol gradient at a flow rate of 1.0 ml/min.

2.2. Animals and sepsis model

Male wild type ICR mice were used as an experimental sepsis model as previously described [14]. For cecal ligation and puncture (CLP), mice were anesthetized with intraperitoneal injections of Zoletil (50 mg/kg) and Rompun (10 mg/kg), after which a small abdominal midline incision was made to expose the cecum. The cecum was then ligated below the ileocecal valve, punctured twice through both surfaces (or once for measurement of cytokine production) using a 22-gauge needle, and the abdomen was closed. Sham CLP mice were subjected to the same procedure but without ligation and puncture of the cecum. Survival was monitored once daily for 10 days.

2.3. Measurement of bactericidal activity in vivo

Twenty-four hours after CLP, peritoneal lavage fluid was collected and cultured overnight on blood-agar base plates (Trypticase Soy Agar Deeps; Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C. The colony forming units (CFUs) were determined as described previously [14].

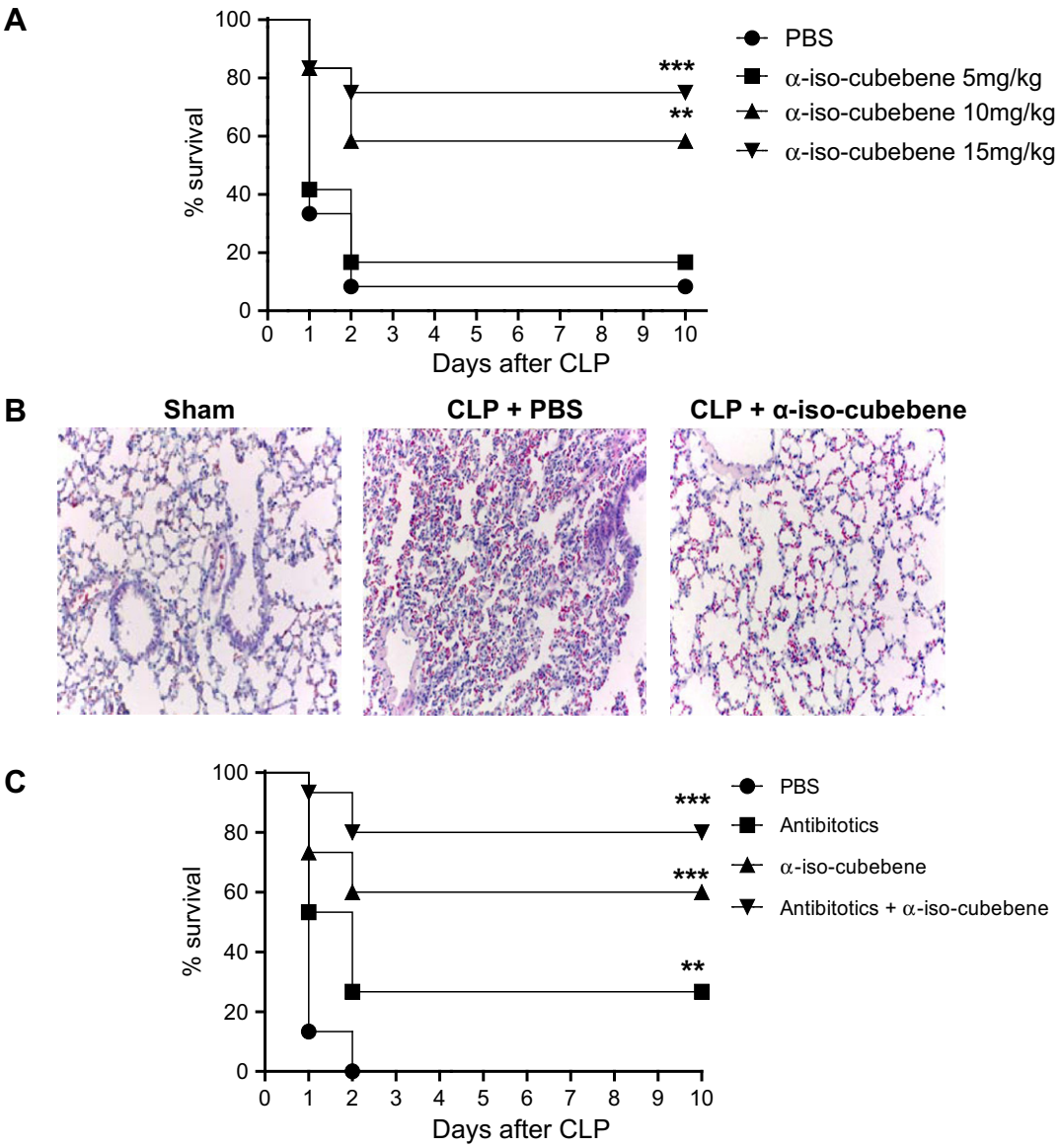

Fig. 1. α-Iso-cubebene protection against CLP-induced mortality. (A) Several doses (0, 5, 10, 15 mg/kg) of α-iso-cubebene were injected subcutaneously four times into CLP mice at 2, 14, 26, and 38 h post-CLP. (B) PBS or α-iso-cubebene (15 mg/kg) was administered 2 and 14 h after CLP. The mice were sacrificed 24 h after surgery and the lungs stained with hematoxylin and eosin (magnification 100×). (C) PBS, α-iso-cubebene (15 mg/kg), antibiotics (8 mg/kg gentamycin plus 8 mg/kg cephalosporin), or α-iso-cubebene plus antibiotics were injected subcutaneously four times into CLP mice at 2, 14, 26, and 38 h post-CLP. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to vehicle control by ANOVA (A, C). Sample size: $n = 15$ mice/group (A, C). The data are representative of eight mice per group (B).

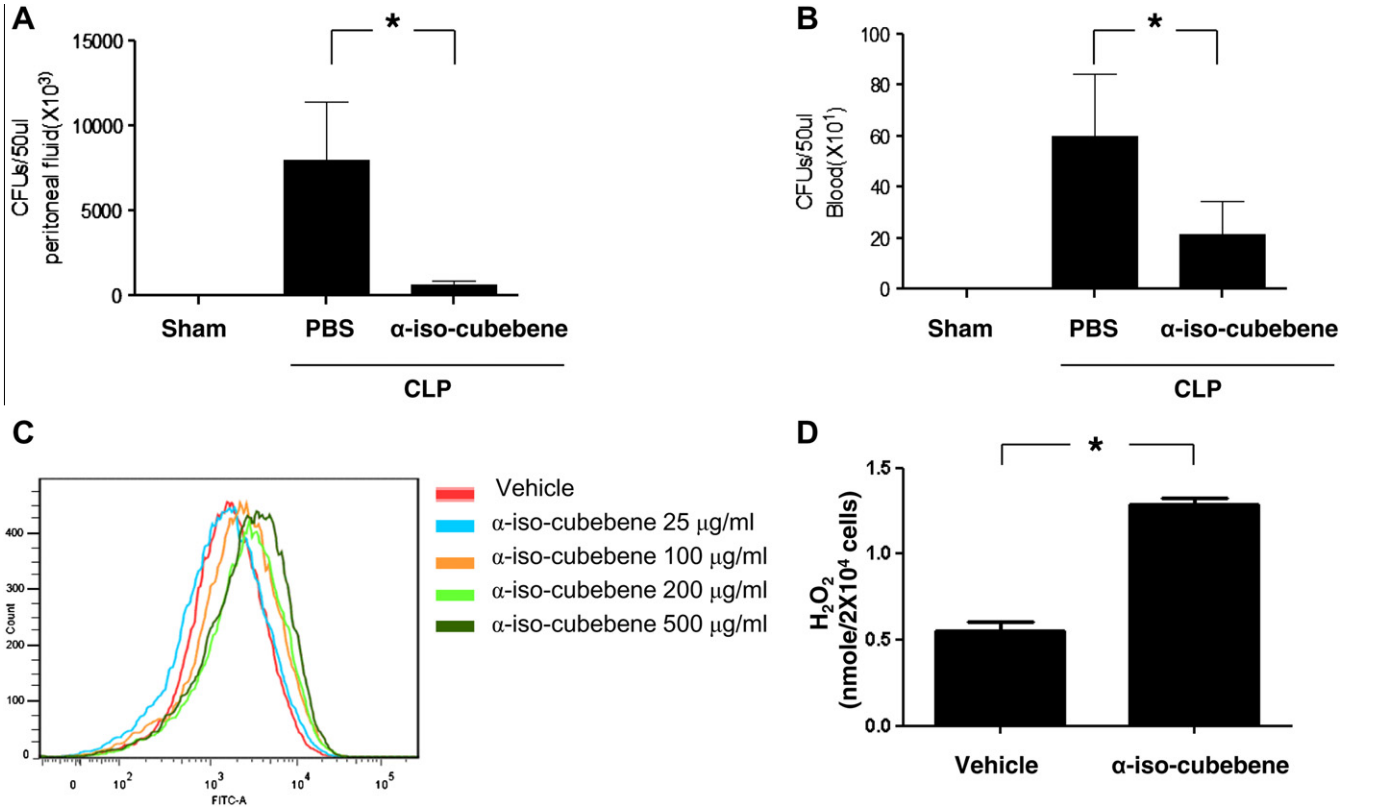

Fig. 2. α-Iso-cubebene enhances bactericidal activity via hydrogen peroxide production. (A) α-Iso-cubebene (15 mg/kg) was injected two times into CLP mice 2 and 14 h post-CLP. Peripheral blood (A), or peritoneal lavage fluid (B) collected 24 h after sham operation or CLP and CLP plus α-iso-cubebene administration was cultured overnight on blood-agar base plates at 37 °C. The CFUs were determined. (C) Raw 264.7 cells (2×10^5) were resuspended in 100 μl PBS and preincubated with or without α-iso-cubebene for 30 min. Then the cells were further incubated with FITC-dextran (1 mg/ml) at 37 °C for 30 min. After fixing the cells, phagocytic uptake was analyzed on a flow cytometer. The result is representative of three independent experiments. (D) Raw 264.7 cells were stimulated with vehicle (PBS) or α-iso-cubebene (500 μg/ml) for 1 h. The amount of hydrogen peroxide produced from the cells was measured using an assay kit. Data are expressed as the mean ± SEM; $n = 8$. $^*P < 0.05$ by t-test.

2.4. Measurement of phagocytic activity

Raw 264.7 cells (2×10^5) were resuspended in 100 μl phosphate-buffered saline (PBS) containing 1% human AB serum and 0.02% sodiumazide, preincubated with or without α-iso-cubebene for 30 min. The cells were further incubated with FITC-dextran (molecular weight, 4 kDa; Sigma-Aldrich, 1 mg/ml) at 37 °C for 30 min. The incubations were stopped by adding 2 ml ice-cold PBS containing 1% human serum. After fixing the cells with 3.7% formaladehyde, phagocytic uptake was analyzed on a flow cytometer (FACS Canto II).

2.5. Tissue histology

Mice were subjected to CLP surgery and given PBS or α-iso-cubebene at a dose of 15 mg/kg 2 h later. The mice were euthanized 24 h after surgery, after which their lungs were fixed, sectioned, and stained with hematoxylin and eosin for morphological analysis.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The TUNEL assay was performed in paraffin-embedded tissue sections, which were first deparaffinized using a standard histological protocol. The sections were then permeabilized with Triton X-100 at 4 °C for 2 min and flooded with terminal deoxynucleotidyl transferase and digoxigenin-dUTP reaction buffer (TUNEL) reagent for 60 min at 37 °C. The percentage of apoptotic (TUNEL-positive) cells was determined by counting 500 splenocytes under a light microscope [15].

2.7. Immunohistochemistry

Immunohistochemistry was conducted using paraffin-embedded tissue sections that were first deparaffinized using a standard histological protocol. After incubation with primary antibodies against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), all sections were stained with horseradish peroxidase-conjugated secondary antibody.

2.8. Cytokine measurement

To measure CLP-induced cytokine production in peritoneal lavage fluid, mice were given α-iso-cubebene at 2, 14, 26, and 38 h after CLP. Peritoneal lavage fluid was collected at various times between 4 and 72 h after CLP, and the cytokines present in the peritoneal fluid were measured by enzyme-linked immunosorbant assay (ELISA; BD Biosciences Pharmingen, San Jose, CA, USA).

2.9. Cytokine release from splenocytes in vitro

Mouse splenocytes (3×10^6 cells/0.3 ml) were placed in RPMI 1640 medium containing 5% fetal bovine serum (FBS) in 24-well plates and kept in a 5% CO_2 incubator at 37 °C. The splenocytes were then incubated with LPS (100 ng/ml) for 6 h in the presence or absence of α-iso-cubebene (25 or 100 μg/ml). LPS (100 ng/ml) was added to the cells 30 min later, and cell-free supernatants

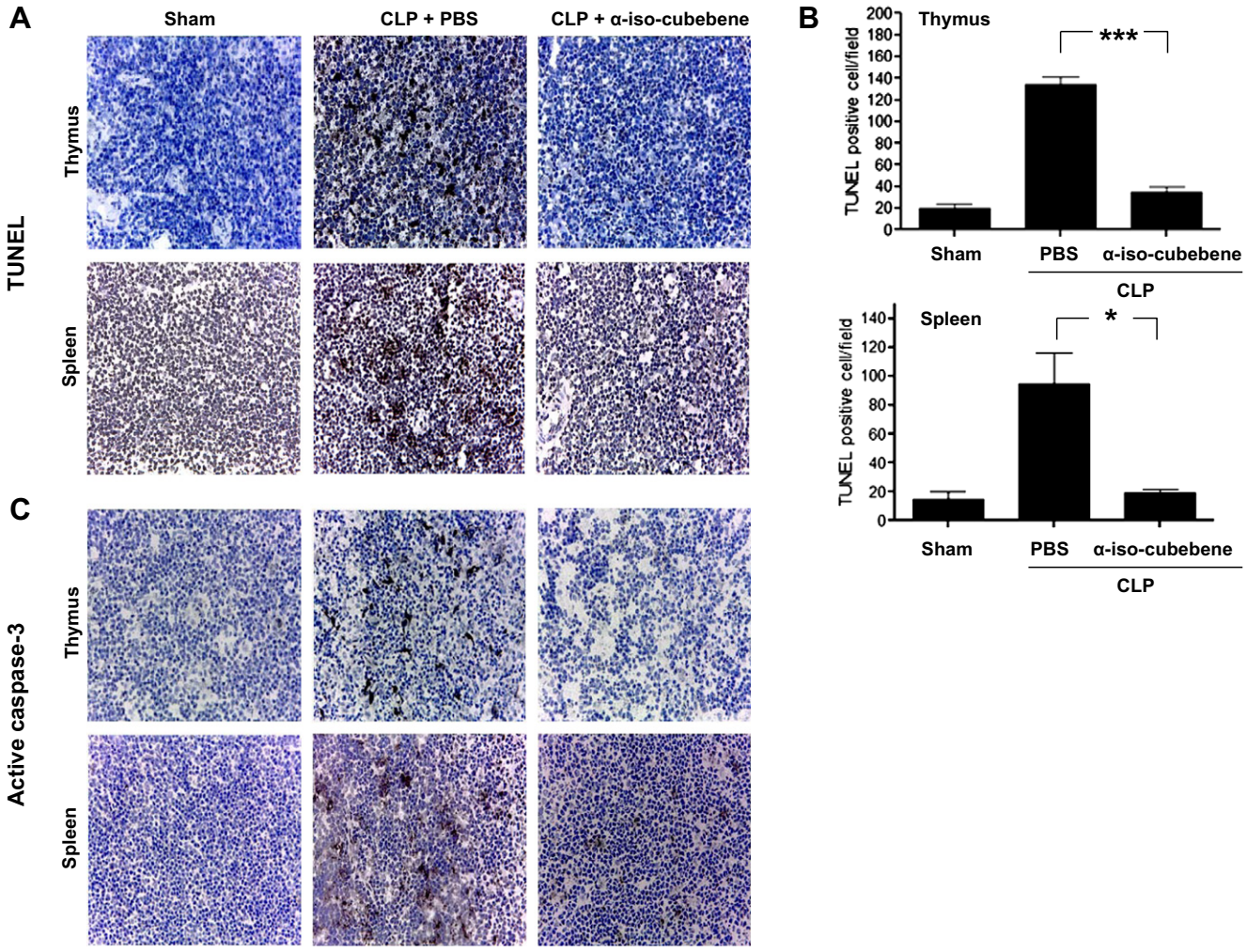

Fig. 3. α-Iso-cubebene protects against widespread CLP-induced leukocyte apoptosis via inhibition of caspase-3 activity. (A) α-Iso-cubebene (15 mg/kg) was injected two times into CLP mice 2 and 14 h post-CLP. The spleen and the thymus collected 24 h after sham, CLP plus PBS, or CLP plus α-iso-cubebene administration was used for TUNEL assay. (B) TUNEL positive cells from spleen and thymus of the mice described in (A) were quantified. (C) The spleens and the thymus from the mice described in (A) were used for immunohistochemistry with cleaved-caspase-3 antibody (magnification 100×). The data are representative of eight mice per group (A, C). Data are expressed as the mean ± SEM ($n = 8$) (B). $^{*}P < 0.05$; $^{***}P < 0.001$ by t-test compared with CLP alone.

were collected, centrifuged, and measured for IL-1β, TNF-α, IL-6, and IL-22 production by ELISA (BD Biosciences Pharmingen) according to the manufacturer's instructions.

2.10. Statistical analyses

Survival data were analyzed using the log-rank test. All other data were evaluated using ANOVA or t-test. The Bonferroni test was used for post hoc comparisons, and statistical significance was set *a priori* at $P < 0.05$.

3. Results and discussion

3.1. Administration of α-iso-cubebene protects against sepsis-induced mortality

To investigate the effect of α-iso-cubebene against polymicrobial sepsis, α-iso-cubebene was subcutaneously administered to CLP mice. α-Iso-cubebene significantly protected against mortality induced by CLP in a dose-dependent manner (Fig. 1A). Survival was strongly enhanced when 10 or 15 mg/kg of α-iso-cubebene was injected 2 h post-CLP and at 12 h intervals three additional times (Fig. 1A).

Sepsis-induced mortality is associated with inflammation of vital organs such as lung [1]. CLP also caused severe alveolar congestion and extensive thrombotic lesions in the lungs, and administration of α-iso-cubebene markedly improved the pulmonary histopathology (Fig 1B).

In the clinical setting, septic patients always receive antibiotics. To test the effect of α-iso-cubebene treatment on survival in mice subjected to CLP in the presence of concomitant treatment with appropriate antibiotic regimen, we administrated α-iso-cubebene in the absence or presence of antibiotics (gentamycin plus cephalosporin). As shown in Fig. 1C, the administration of 8 mg/kg of both antibiotics slightly increased the survival rate in the severe sepsis model. The administration of α-iso-cubebene also slightly enhanced therapeutic effect against severe sepsis. Combination of α-iso-cubebene with the antibiotics additionally increased survival rate (Fig. 1C).

3.2. α-iso-cubebene enhances bacterial clearance

CLP-induced lethality is positively correlated with bacterial colony counts in peripheral blood and peritoneal fluid [1]. Since the

first experiment in the present study showed that α-iso-cubebene enhanced survival against CLP-induced sepsis, we tested whether α-iso-cubebene enhances bacteria killing activity. α-Iso-cubebene enhanced bactericidal clearance in CLP sepsis mice. α-Iso-cubebene treatment almost completely eliminated intraperitoneal bacteria (Fig. 2A) and significantly reduced the number of intravascular bacteria by 80% (Fig. 2B). Bactericidal activity is mediated by phagocytes which engulf bacteria [16]. Accordingly, we also checked whether α-iso-cubebene could affect phagocytic activity of mouse macrophages using fluorescein isothiocynate (FITC)-labeled dextran. Stimulation of Raw 264.7 cells with α-iso-cubebene increased phagocytic activity (Fig. 2C). FITC-positive cells were increased from 47% to 67% by stimulation of the cells with α-iso-cubebene (Fig. 2C). Since reactive oxygen species are well-known weapons for bactericidal activity, we measured the effect of α-iso-cubebene on hydrogen peroxide production from mouse macrophages. α-Iso-cubebene strongly increased the production of hydrogen peroxide in Raw 264.7 cells (Fig. 2D). The results indicate that α-iso-cubebene stimulates bactericidal activity by enhancing phagocytic activity and hydrogen peroxide production in phagocytes.

3.3. α-Iso-cubebene inhibits CLP-induced apoptosis of lymphocytes

Sepsis is associated with cell death of splenocytes and thymocytes with multiple nuclear fragments (apoptotic bodies) in apoptotic lymphocytes. Presently, CLP caused apoptosis of splenocytes and thymocytes. However, the effect was dramatically inhibited by α-iso-cubebene (Fig. 3A). These results were also reflected in DNA fragmentation analysis (TUNEL assay) (Fig. 3A). To quantify the apoptotic cell numbers, TUNEL-positive cells were enumerated. The administration of α-iso-cubebene strongly inhibited CLP-induced apoptosis from the thymus and spleen (Fig. 3B). Similar results were observed when caspase-3 activation was used as a marker of apoptosis in splenocytes and thymocytes. CLP caused substantial caspase-3 activation in splenocytes and thymocytes, which was inhibited by in vivo treatment with α-iso-cubebene (Fig. 3C). Keeping in mind that lymphocyte apoptosis is essentially associated with sepsis-induced organ failure, the observed anti-apoptotic effect of α-iso-cubebene may contribute to increased survival rate in CLP mice.

3.4. Effects of α-iso-cubebene on CLP- or LPS-induced proinflammatory cytokine production

The effect of α-iso-cubebene on CLP-induced cytokine production in peritoneal fluid was measured 24 h after CLP (Figs. 4A–D). CLP induced a dramatic increase in the production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 within 24 h. Treatment with α-iso-cubebene significantly decreased TNF-α, IL-1β, and IL-6 levels (Figs. 4A–C). In addition to the well-known proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, IL-22 has been suggested to mediate inflammatory response in sepsis [17]. In this study we found that the level of IL-22 was significantly decreased by injection of α-iso-cubebene (Fig. 4D).

Since α-iso-cubebene showed therapeutic effect against CLP sepsis by reducing the production of proinflammatory cytokines, the next experiment examined whether α-iso-cubebene could act directly on leukocytes to inhibit LPS-stimulated proinflammatory cytokine production. Stimulation of freshly isolated splenocytes with 100 ng/ml LPS (as a prototypical microbial signal) increased of levels of TNF-α, IL-1β, IL-6, and IL-22, which were significantly decreased in α-iso-cubebene-treated cells compared to LPS only treated cells (Figs. 4E–H).

The collective results demonstrate that the therapeutic administration of α-iso-cubebene after induction of sepsis by CLP can effectively inhibit CLP-induced lethality in mice. Mechanistically α-iso-cubebene showed therapeutic activity against an experimental animal model of sepsis by at least three different mechanisms: (1) increase in bactericidal activity, (2) inhibition of leukocyte apoptosis, and (3) inhibition of proinflammatory cytokine secretion.

Previously, we reported that α-iso-cubebene inhibits inflammatory response by inhibiting TNF-α-stimulated response in endothe-

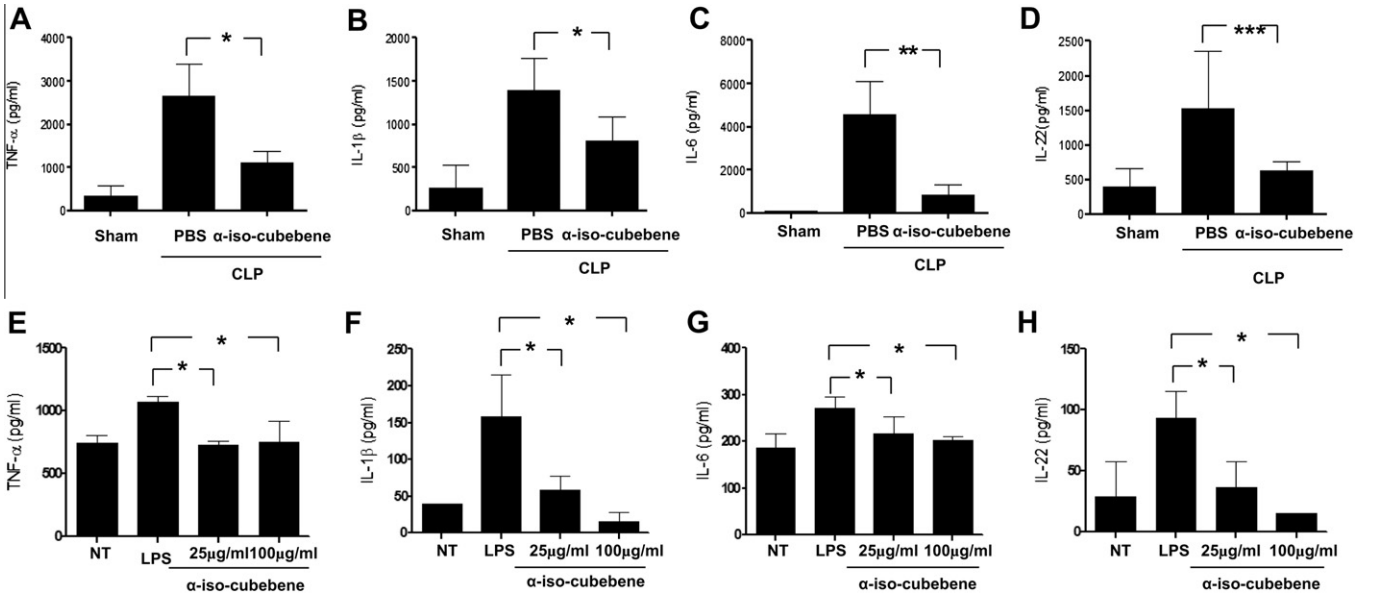

Fig. 4. Effect of α-iso-cubebene on CLP- or LPS-induced cytokine production. (A–D) α-Iso-cubebene (15 mg/kg) was injected four times into CLP mice at 2, 14, 26, and 38 h post-CLP. Separate groups of animals were given sham, CLP plus PBS, or CLP plus α-iso-cubebene treatment. Peritoneal fluids were collected at several different times after CLP. The amounts of cytokines in the peritoneal fluid were determined by ELISA analysis. Panels A–D display results for TNF-α, IL-1β, IL-6, and IL-22 respectively. (E–H) Mouse splenocytes were pre-incubated with PBS or α-iso-cubebene (25 or 100 μg/ml) for 30 min and then stimulated with PBS or LPS (100 ng/ml) for 3 h. The levels of TNF-α (F), IL-1β (E), IL-6 (G), and IL-22 (H) were measured by ELISA. Data are expressed as the mean ± SEM ($n = 8$ for A–H). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by t-test compared with CLP alone or LPS alone.

lial cells [13]. Presently, we demonstrated that α-iso-cubebene induced a dramatic change in the levels of several cytokines in the early stages of the CLP model. Proinflammatory cytokine (IL-1β and TNF) levels were decreased by α-iso-cubebene treatment (Fig. 4). Others have shown that decreases in certain proinflammatory cytokines, such as IL-1β and TNF, contribute to an increase in survival in sepsis models [18–20].

Since α-iso-cubebene further increased anti-septic activity in the treatment of antibiotics regimen, the collective results support the potential of α-iso-cubebene as a useful material for the development of therapeutic drugs against sepsis and microbial infectious diseases.

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