Article

Albaconol, a Plant-Derived Small Molecule, Inhibits Macrophage Function by Suppressing NF-κB Activation and Enhancing SOCS1 Expression

Qiuyan Liu[1, 4], Xiaoli Shu[2, 4], Li Wang[2], Anna Sun[2], Jikai Liu[3, 5] and Xuetao Cao[1, 2, 5]

Discovery and functional identification of plant-derived small compounds as the immunosuppressant attract much attention these years. Albaconol is a new kind of small compound, prenylated resorcinol, isolated from the fruiting bodies of the inedible mushroom *Albatrellus confluens*. Our previous studies showed that albaconol can inhibit tumor cell growth and dendritic cell maturation. However, the immunomodulatory roles and the underlying mechanisms of albaconol have not been fully understood. In this study we investigated the effects of albaconol on the proliferation and LPS-induced proinflammatory cytokine production of macrophages. Albaconol, when used at a dose higher than 1.0 μg/ml, inhibited proliferation of RAW264.7 cells in a dose- and time-dependent manner, and could induce cellular apoptosis when used at high dosage (≥ 7.5 μg/ml). Furthermore, we found that albaconol used at a lower dosage without apoptosis induction could significantly inhibit LPS-induced TNF-α, IL-6, IL-1β and NO production in RAW264.7 cells. The inhibition of NF-κB activation and enhancement of SOCS1 expression in LPS-stimulated macrophages by albaconol may contribute to the above immunosuppressive or anti-inflammatory activities of albaconol. Our results suggest that albaconol may be a potential immunosuppressive and anti-inflammatory drug. *Cellular & Molecular Immunology*. 2008;5(4):271-278.

Key Words: immunosuppressant, macrophage, proinflammatory cytokine, NF-κB, SOCS1

Introduction

Some kinds of plant-derived natural small molecules have been found to be immunosuppressive, and are now widely used in the treatment of inflammation and autoimmune diseases. For example, several substances with immunomodulatory and/or antitumor activities have been isolated from mushrooms (1), including polysaccharides and polysaccharopeptides. Furthermore, other bioactive substances, including triterpenes, lipids and phenols, have been identified and characterized in mushrooms with proven medicinal properties. The biological activities of these active substances derived from mushrooms include mitogenicity and activation of immune cells, such as hematopoietic stem cells, lymphocytes, macrophages, dendritic cells and NK cells, resulting in the production of cytokines. As part of a search for naturally occurring bioactive metabolites of the higher fungi and for analogues of the scutigeral type, the chemical composition of mushroom *Albatrellus confluen* was investigated by us (2). We found that mushroom *Albatrellus confluens* contained high concentrations of albaconol, a new prenylated resorcinol. We demonstrated that albaconol, as a partial agonist of the vanilloid receptor, could induce contraction and desensitization of guinea pig trachea *in vitro* (3). Furthermore, we showed that albaconol could inhibit the growth of human tumor cell lines through its influence on the DNA topoisomerases,

[1]Institute of Immunology and National Key Laboratory of Medical Immunology, Second Military Medical University, Shanghai 200433, China;

[2]Institute of Immunology, Zhejiang University, Hangzhou 310031, China;

[3]State Key Laboratory of Phytochemistry and Plant Resourses in West China, Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming 650204, China;

[4]Qiuyan Liu and Xiaoli Shu contributed equally to this work;

[5]Corresponding to: Dr. Xuetao Cao, Institute of Immunology and National Key Laboratory of Medical Immunology, Second Military Medical University, Shanghai 200433, China. Fax: +86-21-6538-2502, E-mail: caoxt@public3.sta.net.cn; or Dr. Jikai Liu, State Key Laboratory of Phytochemistry and Plant Resourses in West China, Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming 650204, China. E-mail: jkliu@mail.kib.ac.cn

Received Jul 7, 2008. Accepted Jul 15, 2008.

Abbreviations: DMSO, dimethyl sulfoxide; IL-1β, interleukin 1β; IL-6, interleukin 6; IκB, inhibitior of κB; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SOCS1, suppressor of cytokine signalling-1; NO, nitric oxide; TNF-α, tumor necrosis factor α.

indicating that albaconol may exhibit multiple biological activities including potential antitumor effects (4). However, the roles and the underlying mechanisms of the plant-derived albaconol in the regulation of immune response and inflammation need to be investigated.

The findings that exogenous vanilloids are involved in the regulation of immune response (5) and the inhibitory effect of albaconol on the cellular growth indicates that albaconol may exhibit immunoregulatory effects. One of previous studies showed that albaconol could inhibit LPS-induced expression of MHC-II and costimulatory molecules, and T cell-stimulating capacity of DCs (6). However, there is no report about the regulation of macrophage functions by albaconol. Considering that macrophages play important roles in the inflammation, and macrophages-derived TNF-α, IL-1β, IL-6 and NO are important proinflammatory cytokines and mediators, in this study, we investigated the effects of albaconol on the proliferation of macrophages and the production of TNF-α, IL-1β, IL-6 and NO by LPS-treated macrophages. Furthermore, we demonstrated that albaconol could attenuate LPS-induced production of proinflammatory mediators in macrophages through inhibiting NF-κB activation and up-regulating SOCS1 expression.

Materials and Methods

Reagents

Albaconol was isolated and identified from mushroom *Albatrellus confluens* as described previously (2). Albaconol (> 99%, HPLC, MW: 346) was dissolved in DMSO at 5 mg/ml as stock solution and stored at -20°C. DMSO contained in the maximal tested concentration (albaconol 7.5 μg/ml) was 0.15%. The stock solution was diluted with medium to the test concentration just before use. Media and DMSO control wells, in which albaconol was absent, were included in all the experiments in order to eliminate the influence of DMSO. 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was from Bio-Light (Shanghai, China). Annexin V Apoptosis Detection Kit and Griess Reagent were purchased from Sigma-Aldrich (NSW, Australia). ELISA kits for murine TNF-α, IL-1β and IL-6 were from R&D Systems (Minneapolis, MN). M-PER® Mammalian Protein Extraction Reagent, NE-PER® Nuclear and Cytoplasmic Extraction Reagents, BCA™ Protein Assay Kit and SuperSignal® West Femto Maximum Sensitivity Substrate were purchased from Pierce (Rockford, IL). pGL3.5×κB-luciferase plasmid was kindly provided by Seamus J. Martin (6), and pRL-TK-Renilla-luciferase plasmid was purchased from Promega (Madison, WI). Monoclonal antibody against actin, NuP62 and polyclonal antibodies (Abs) against phosphor-IκB-α (Ser32), NF-κB p65 (Ser536), iNOS and SOCS1 were from Cell Signaling Technology (Beverly, MA). DMSO and LPS were from Sigma (*Escherichia coli*, O26:B6, St. Louis, MO).

Cell line and culture

The murine macrophage cell line RAW264.7 was from ATCC (American Type Tissue Culture Collection), and maintained in DMEM supplemented with 10% fetal calf serum at 37°C in 5% CO_2.

Cell proliferation assay

Cell proliferation was detected by MTT method. Briefly, RAW264.7 cells were cultured in 96-well plates overnight, and then treated with indicated amounts of albaconol for 24 h or with albaconol for indicated time periods. Finally, 10 μl/well MTT (5 mg/ml) was added to cell culture system 4 h before the end of culture. Then, the MTT-containing medium was removed and cells were lysed by 150 μl/well of DMSO. Absorbance was assessed spectrophotometrically at 570 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA). The inhibitory rate of cell proliferation was calculated by the following formula: Growth inhibition (%) = $[(OD_{DMSO} - OD_{albaconol}) / OD_{DMSO}] \times 100\%$. The cytotoxicity of albaconol on RAW264.7 cells was expressed as IC_{50} values (the drug concentration reducing by 50% the absorbance in treated cells, with respect to untreated cells) and was calculated by LOGIT method. Results were obtained from more than three independent experiments.

Detection of cellular apoptosis

RAW264.7 cells were treated with the indicated amounts of albaconol. Twenty-four hours later, cells were harvested and labeled with Annexin V-FITC and propidium iodide (PI) following manufacturer's instructions. Samples were examined by FACS, and the data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA) as described previously (7).

RT-PCR analysis of cytokine expression

After treatment with or without 5 μg/ml albaconol for 24 h, RAW264.7 cells were stimulated with LPS (100 ng/ml) for 12 h, then washed and harvested. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and single-strand cDNA was synthesized from 2 μg of total RNA using AMV reverse transcriptase (Promega, Madison, WI). The primers of murine TNF-α (forward 5'-GAC ACC ATG AGC CAA AAG-3', reverse 5'-GAG TAG ACA AGG TAC AAC CC-3'), IL-1β (forward 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3', reverse 5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3'), IL-6 (forward 5'-GAG AGG AGA CTT CAC AGA GGA TAC-3', reverse 5'-GTA CTC CAG AAG ACC AGA GG-3'), iNOS (forward 5'-ATG GCT TGC CCC TGG AAG TTT CTC-3', reverse 5'-CCT CTG ATG GTG CCA TCG GGC ATC TG-3') and GAPDH (forward 5'-ACC ACA GTC CAT GCA TCA C-3', reverse 5'-TCC ACC ACC CTG TTG CTG TA-3') were designed using the software Primer Preimer 5.0 and synthesized by Sangon Inc (Shanghai, China). The cDNA pre-denaturation involved one cycle of 94°C for 2 min, immediately followed by 25 cycles (GAPDH) or 32 cycles of 30 s at 94°C (dissociation), 56°C (primer annealing), and 72°C (extension), and finally a 10 min incubation at 72°C to complete the primer extension. Oligonucleotide primers for GAPDH were used as control as

described previously (8).

Assays for cytokines and NO

RAW264.7 cells were treated with the indicated amounts of albaconol for 24 h. After subsequently stimulated with 100 ng/ml LPS for 24 h, the supernatants were harvested. The concentrations of TNF-α, IL-1β and IL-6 in culture supernatants were measured using mouse TNF-α, IL-1β and IL-6 ELISA Kits. The concentrations of NO in supernatants were measured using the Griess reagent as described previously (9).

Western blotting

RAW264.7 cells treated with albaconol for 24 h were subsequently stimulated with 100 ng/ml LPS for the indicated time. After being washed twice with cold PBS, cells were lysed with M-PER™ Protein Extraction Reagent containing protease inhibitor (Calbiochem) and phenylmethanesulfonyl fluoride (PMSF) or were separated into cytoplasmic and nuclear extracts by NE-PER® Nuclear and Cytoplasmic Extraction Reagents. Protein concentrations of the extracts were measured by BCA assay. Equal amounts of protein were subjected to SDS-PAGE, and then transferred onto nitrocellulose membranes as described previously by us (10). The level of iNOS and SOCS1 expression, degradation of IκB-α and nuclear translocation of the p65 subunit of NF-κB were studied by Western blot analysis. Actin level in total cell lysates or cytoplasmic extraction and NuP62 in nuclear extraction were detected to show equal protein loading.

Dual-luciferase reporter assay

RAW264.7 cells were co-transfected with the mixture of 100 ng pGL3.5×κB-luciferase reporter plasmid, 10 ng pRL-TK-*Renilla*-luciferase plasmid using Lipofectimine™ 2000 Reagent (Invitrogen, Carlsbad, CA) as described previously by us (11). Forty-eight hours after transfection, cells were left untreated or treated with indicated concentrations of albaconol. NF-κB luciferase activities in cell lysates were measured using Dual-Luciferase Reporter Assay System. Data were normalized for transfection efficiency by dividing *Firefly* luciferase activity with that of *Renilla* luciferase. The relative values are presented as fold decrease over indicated control. All transfection experiments were done in triplicate wells and repeated separately at least 3 times.

Statistical analysis

Data were represented as mean ± standard deviation (SD) of more than three separate experiments performed in triplicate. Statistical significance between any two groups was determined by two-tailed Student's *t* test. *p*-values less than 0.05 were considered to be significant.

Results

Albaconol inhibits proliferation of macrophages in a dose- and time-dependent manner

To investigate the potential effects of albaconol on the proliferation and survival of macrophages, RAW264.7 cells were treated with various concentrations of albaconol (0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 μg/ml) for 24 h, respectively. As shown in Figure 1A, albaconol could significantly inhibit proliferation of RAW264.7 cells, with more potent inhibition when the higher concentration of albaconol used. The IC_{50} value was 5.55 ± 0.4 μg/ml. Then we used 2.5 and 5.0 μg/ml albaconol to treat RAW264.7 cells for the indicated times (8, 12, 24, 33, 47 and 53 h), and found that the proliferation of RAW264.7 cells was inhibited obviously after 24 h and the inhibitory effects were maintained well from 24 h to 47 h (Figure 1B). Therefore, albaconol can inhibit proliferation of macrophages in a dose- and time-dependent manner.

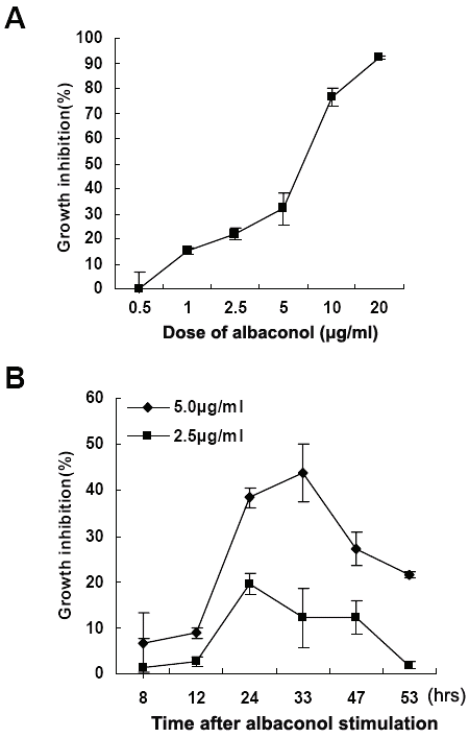

Figure 1. Albaconol inhibits proliferation of macrophages. RAW264.7 cells (1.5×10^3) were (A) treated with albaconol at various concentrations (0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 μg/ml) for 24 h respectively, or (B) treated with 2.5 and 5.0 μg/ml albaconol for the indicated times (8, 12, 24, 33, 47 and 53 h). Proliferation of RAW264.7 cells was examined by MTT assay and the percentage of cell growth inhibition was calculated according to the formula given in Materials and Methods. Data represented means ± SD of triplicate. The shown data was representative of three independent experiments with similar results.

Induction of macrophage apoptosis by albaconol

To study whether albaconol inhibited proliferation of macrophages by apoptosis induction, RAW264.7 cells were treated with albaconol at different concentrations for 24 h and their apoptosis was analyzed by Annexin V and PI double staining. As shown in Figure 2, 7.5 μg/ml albaconol could induce apoptosis of RAW264.7 cells, but there was no obvious

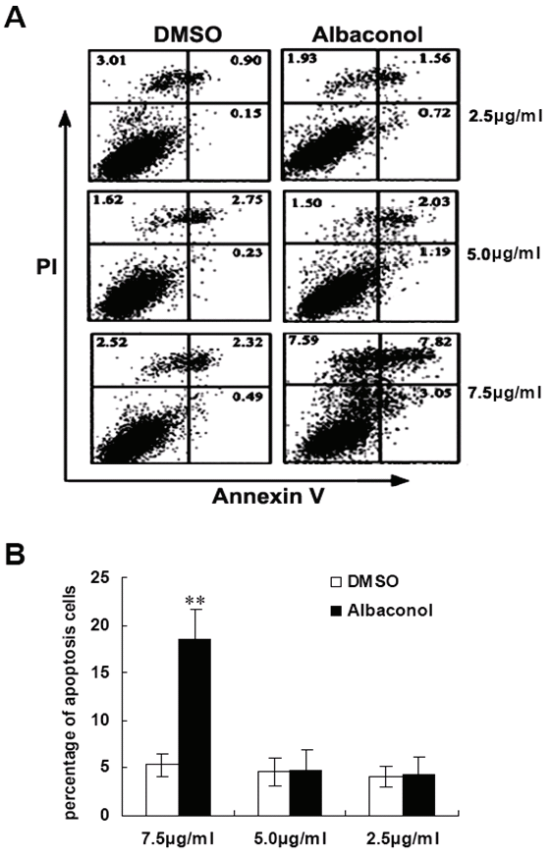

Figure 2. Albaconol at high concentration induces apoptosis of macrophages. RAW264.7 cells (1×10^5) in 24-well plates were treated with albaconol (7.5, 5.0, and 2.5 μg/ml) for 24 h, and DMSO was used as control. Cells were harvested and the percentage of apoptotic cells was determined by FACS analysis using Annexin V/PI staining. The results are representative of three independent experiments (A). Apoptosis was represented by the percentage of cells which were Annexin V and/or PI-positive, and results were presented as means ± SD from three independent experiments (B).

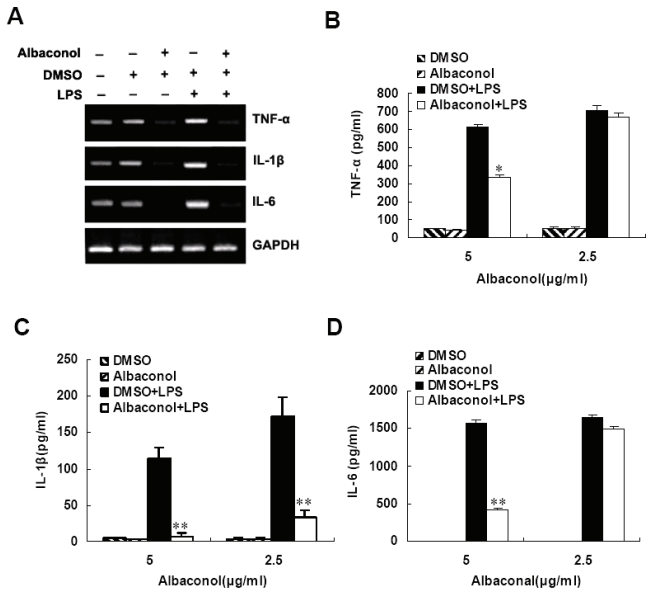

Figure 3. Inhibitory effect of albaconol on the production of proinflammatory cytokines in macrophages. (A) RAW264.7 cells (5×10^5) in 6-well plates were treated with albaconol (5.0 μg/ml) for 24 h. DMSO (0.25%, v/v) was used as control. Then the RAW264.7 cells were stimulated with or without 100 ng/ml LPS for 24 h. Total RNA was prepared from each sample and RT-PCR was performed to analyze the mRNA expression of cytokines TNF-α, IL-1β and IL-6. (B-D) RAW264.7 cells (1×10^5) in 24-well plates were treated with albaconol (5.0 and 2.5 μg/ml) for 24 h. Then cells were stimulated with or without 100 ng/ml LPS for 24 h. TNF-α, IL-6 and IL-1β in supernatants were assayed. Results were mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ for the comparison of LPS-stimulated cells treated with DMSO only.

proapoptotic activity of albaconol observed when used at the doses of 5.0 μg/ml and 2.5 μg/ml. The results suggested that the inhibitory effect of albaconol at the dose higher than 5.0 μg/ml on the macrophage proliferation may be, at least partially, due to apoptosis induction, however, there exist other unknown mechanisms by which albaconol at lower concentration inhibits proliferation of macrophages. So, we selected the lower concentration of albaconol at 2.5 μg/ml and/or 5.0 μg/ml to observe the immunomodulatory effect of albaconol in the following experiments.

Albaconol inhibits the production of TNF-α, IL-1β and IL-6 in LPS-induced macrophages

First, mRNA expression of proinflammation cytokines including TNF-α, IL-1β and IL-6 in RAW264.7 cells with or without LPS stimulation, in the presence or absence of albaconol, was evaluated by RT-PCR (Figure 3A). Albaconol (5.0 μg/ml) significantly inhibited mRNA expression of these proinflammatory cytokines in both control RAW264.7 cells and LPS (100 ng/ml)-stimulated RAW264.7 cells, suggesting that albaconol may inhibit production of these proinflammatory cytokines. Next, we found that albaconol at the dose of 5.0 μg/ml obviously inhibited LPS-induced production of TNF-α, IL-1β and IL-6. However, albaconol at 2.5 μg/ml inhibited the production of IL-1β but not TNF-α and IL-6 (Figures 3B-3D). Therefore, albaconol, at lower concentrations without apoptosis induction, can inhibit the production of TNF-α, IL-1β and IL-6 in LPS-induced macrophages, indicating that albaconol may exhibit anti-inflammatory and immunosuppressive activities.

Albaconol inhibits LPS-induced iNOS expression and NO production in macrophages

Since NO is also known as a proinflammatory mediator in many different acute and chronic inflammatory diseases, we then addressed whether albaconol modulates NO production from the macrophages activated by LPS. RAW264.7 cells were pretreated with albaconol at various concentrations for 24 h and subsequently treated with 100 ng/ml LPS. As shown in Figure 4B, albaconol at the concentration of 5.0 μg/ml markedly inhibited LPS-induced production of nitrite, a

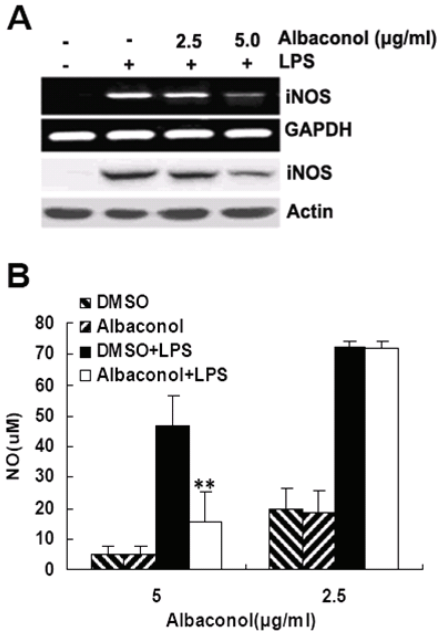

Figure 4. Albaconol inhibits LPS-induced iNOS expression and NO production in macrophages. RAW264.7 cells (1×10^5) in 24-well plates were treated with albaconol (5.0 and 2.5 μg/ml) for 24 h. Then the RAW264.7 cells were stimulated with or without 100 ng/ml LPS for 12 h for RT-PCR assay of iNOS expression and 24 h for Western blot assay of iNOS expression. (A) iNOS expression was detected by PT-PCR (upper panels) and Western blot (lower panels), with GAPDH and actin as the loading control. (B) NO in supernatant was assayed using the Griess reagent. Results were mean ± SD of three independent experiments. $^{**}p < 0.01$ for the comparison of LPS-stimulated cells treated with DMSO only.

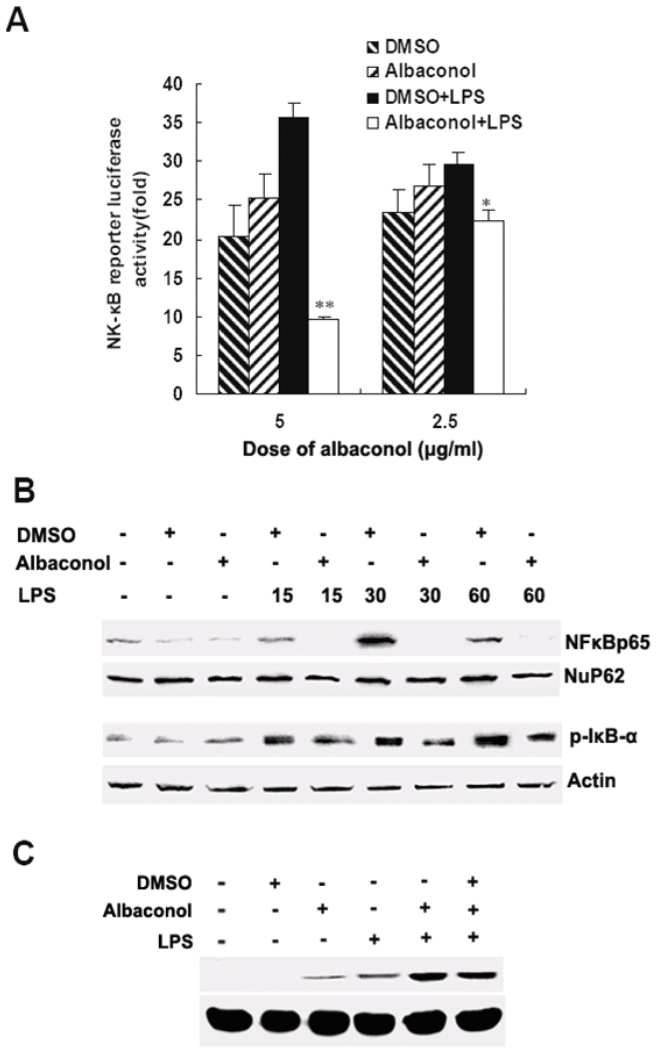

Figure 5. Albaconol inhibits LPS-induced NF-κB activation and enhances SOCS1 expression in macrophages. (A) RAW264.7 cells (1.0×10^4) in 96-well plates were co-transfected with NK-κB reporter luciferase plasmid and TK-Renilla plasmid for 48 h and the transfected cells were treated with albaconol (5.0 and 2.5 μg/ml), or DMSO for 24 h, then the cells were activated by LPS (100 ng/ml) or not for 6 h. Luciferase activities were measured using Dual-Luciferase Reporter Assay System. Results were normalized for transfection efficiency by dividing *Firefly* luciferase activity with that of *Renilla* luciferase. The relative values are presented as fold increase over indicated control. $^{*}p < 0.05$, $^{**}p < 0.01$ for the comparison of LPS-stimulated cells treated with DMSO only. (B) RAW264.7 cells (5×10^5) in 6-well plates were treated with albaconol (5.0 μg/ml) for 24 h, or with DMSO (0.25%, v/v) as control. Then the RAW264.7 cells were stimulated with or without LPS (100 ng/ml) for 15, 30 and 60 min. Cells were collected and proteins were extracted by NE-PER® Nuclear and Cytoplasmic Extraction Reagents. NF-κB p65 in nuclear extract and phosphorylation of IκB-α were detected by Western blotting. (C) RAW264.7 cells (5×10^5) in 6-well plates were treated with albaconol (5.0 μg/ml and 2.5 μg/ml) for 24 h, or with DMSO as control. Then the RAW264.7 cells were stimulated with or without LPS (100 ng/ml) for 24 h. SOCS1 was detected by Western blot. Reproductive results were obtained in three independent experiments with similar results.

stable oxidized product of NO. However, no inhibitory effect of albaconol at 2.5 μg/ml on NO production was observed, which was consistent with the findings that albaconol at the same concentration had no effect on the LPS-induced production of TNF-α and IL-6 in macrophages. Furthermore, we assessed the effect of albaconol on iNOS expression, as shown in Figure 4A. Unstimulated RAW264.7 cells did not contain iNOS, whereas addition of 100 ng/ml LPS induced iNOS synthesis, and albaconol at 5.0 μg/ml markedly inhibited LPS-induced iNOS expression, but no inhibitory effect of albaconol at 2.5 μg/ml was observed, which was consistent with NO production.

Inhibition of NF-κB activity and IκB-α degradation by albaconol in LPS-stimulated macrophages

MAPK (ERK, JNK and p38) and NF-κB are known to be associated with proinflammatory cytokine gene expression and play important roles in TLR4 signaling pathway (12-14). However, albaconol treatment does not affect LPS-induced phosphorylation of these signaling components of the MAPK signaling cascade (p-ERK, p-p38, p-JNK), indicating MAPK pathway may be not involved in the above suppression (data not shown). We further investigated the effect of albaconol

on LPS-induced NF-κB activation in macrophages by detecting NF-κB luciferase reporter gene expression. RAW264.7 cells were transiently co-transfected with NK-κB reporter luciferase plasmid and TK-Renilla plasmid, then treated with indicated concentration of albaconol for 24 h, following stimulation with 100 ng/ml LPS. Exposure of RAW264.7 cells to LPS increased reporter activity by about 2.5 fold compared with that of groups without LPS stimulation. Albaconol dose-dependently reduced the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (0.3-0.7 fold as compared with that of DMSO-treated control group), as shown in Figure 5A.

Activation of NF-κB involves two important steps: (a) phosphorylation and subsequent degradation of IκB-α caused by IκB kinase, resulting in release of NF-κB, and (b) the nuclear translocation of the activated NF-κB. To gain further insight into mechanism of albaconol-mediated inhibition of NF-κB activation, we examined the effect of albaconol on IκB-α degradation and nuclear translocation of NF-κB in RAW264.7 cells. As shown in Figure 5B, the phosphorylation levels of IκB-α protein in cytoplasm and the nuclear level of p65 subunit of NF-κB were up-regulated in RAW264.7 cells after LPS stimulation for 15 min and lasted 60 min. However, treatment of RAW264.7 cells with albaconol prior to LPS stimulation decreased IκB-α phosphorylation level and NF-κB (p65) nuclear content. These data indicated that albaconol could inhibit LPS-induced NF-κB activation in macrophages through interfering with nuclear translocation and transcription activity of NF-κB.

Albaconol up-regulates LPS-induced SOCS1 expression in macrophages

Suppressor of cytokine signaling (SOCS) proteins have been identified as negative feedback inhibitors for various cytokines signaling *via* the JAK/STAT pathway. SOCS1 interacts with JAK tyrosine kinases and inhibits the kinase activity, thereby suppressing the cytokine signal transduction (15). Recently, SOCS1 was found to be induced by LPS stimulation in macrophages (16) and it is reported that SOCS1 is an essential down-regulating factor for protecting host from fatal responses to LPS (17). Particularly, SOCS1 overexpression suppressed LPS-induced IκB phosphorylation and NF-κB transcriptional activity (18). Therefore, to determine if albaconol could induce SOCS1 expression to suppress LPS signaling, we analyzed LPS-induced expression of SOCS1 in RAW264.7 cells pretreated with albaconol. The results in Figure 5C showed that albaconol could increase LPS-induced SOCS1 expression markedly. These results suggested that SOCS1 maybe involved in the inhibitory effects of albaconol on the LPS-induced production of pro-inflammatory cytokines and NF-κB activation in macrophages.

Discussion

In the present study, we demonstrate the immunomodulatory effects of a novel natural small compound albaconol on the macrophages, by showing that albaconol suppresses proliferation of macrophages and LPS-induced production of proinflammatory mediators in macrophages. We found that albaconol inhibited proliferation of RAW264.7 cells in a dose- and time-dependent manner. The production of TNF-α, IL-1β, IL-6 and NO in LPS-stimulated RAW264.7 cells was suppressed by albaconol at lower concentrations without apoptosis induction. These results suggest that albaconol may be a candidate of immunosuppressive and anti-inflammatory drugs.

One of our previous studies showed that albaconol significantly inhibited the growth of human chronic myelogenous leukemia cell line K562, human lung adenocarcinoma cell line A549, human gastric adenocarcinoma cell line BCG-823 and human breast carcinoma cell line Bcap-37 in a dose-dependent manner, and albaconol targeted specifically to DNA topo II, which may be one of the mechanisms of its antitumor action (4). Many kinds of immunosuppressive drugs were once used as drugs with antitumor activity, such as cyclophosphamide, methotrexate. Therefore, we examined the immunosuppressive effect of albaconol on the macrophages by using the murine macrophage cell line RAW264.7 as a cellular model. Interestingly, albaconol inhibited the proliferation of RAW264.7 cells obviously in a dose- and time-dependent manner. Although albaconol at a higher dosage could induce apoptosis of RAW264.7 cells, the anti-proliferative action of albaconol was probably mediated in a cytostatic rather than a cytotoxic manner, because albaconol at lower dosages without apoptosis induction did inhibit the growth of RAW264.7 cells obviously.

The production of these inflammatory molecules by RAW264.7 cells can be induced markedly in response to LPS stimulation (19, 20). Our results provided valuable information that albaconol decreased the LPS-induced production of TNF-α, IL-1β and IL-6 in macrophages. In addition to the inhibitory effects on proinflammatory cytokines production, albaconol also significantly attenuated the secretion of NO of macrophages induced by LPS. The small amount of NO produced by constitutive NOS, including endothelial NOS and neural NOS, is an important regulator of physiological homeostasis, whereas the large amount of NO produced by inducible NOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation (21). NO is induced by LPS or immunological stimuli (e.g., IFN-γ) implicating as a mediator of inflammation (22). It has been well known that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to septic shock, cerebral injury, myocardical ischemia, local or systemic inflammatory disorders, diabetes and other diseases. Therefore, inhibition of NO is also potentially beneficial. The inhibition of TNF-α, IL-1β, IL-6 and NO production by albaconol suggests that albaconol is involved in the down-regulation of late or prolonged production of proinflammatory mediators and may contribute to anti-inflammation and immunosuppression.

To date, several important common pathways have been

identified in the molecular mechanisms by which compounds and fractions inhibit the proinflammatory responses. One of these common pathways is known to be NF-κB, as it controls the expression of proinflammatory genes such as cytokines, adhesion molecules and cytotoxic molecule-generating enzymes including iNOS and COX-2 (23, 24). So, many researchers are trying to develop potent inhibitors of NF-κB as a novel anti-inflammatory drug. Indeed, some strong NF-κB inhibitors, such as sesquiterpene lactone compounds (25-27), display a broad spectrum of inhibition on the expression of proinflammatory genes. Our data elucidated that NF-κB may be one of the targets of albaconol, because their inhibitory patterns on the production of cytokines (as well as NO) were apparently consistent with the inhibition of NF-κB activity induced by LPS in RAW264.7 cells. It's well known that the mitogen-activated protein (MAP) kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses (28). However, no effect of albaconol on the LPS-induced MAPK pathway was observed (data not shown) and it seems to indicate that MAP kinases are not involved in the anti-inflammatory effect of albaconol in macrophages.

In addition, Jak2 and Stat5 were found to be involved in LPS-induced IL-6 production and that SOCS-1 selectively inhibited LPS-induced IL-6 production by regulating Jak2 and Stat5 activation (29). LPS-induced IκB and p38 phosphorylation was up-regulated in SOCS1$^{-/-}$ macrophages and forced expression of SOCS1 suppressed LPS-induced NF-κB activation (18). Since the expressions of proinflammatory mediators and the activity of NF-κB are known to be modulated by SOCS1, we found that albaconol could induce SOCS1 up-regulation in RAW264.7 cells stimulated by LPS, thus outlining one of mechanistic explanation for the anti-inflammatory effect of albaconol.

In conclusion, we demonstrate that albaconol is a potent inhibitor of the LPS-induced TNF-α, IL-1β, IL-6 and NO production in macrophages through suppression of NF-κB activation and up-regulation of SOCS1 expression. These findings suggest that albaconol is a potential candidate of immunosuppressive and anti-inflammatory drugs.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (30721091) and the National Key Basic Research Program of China (2007CB512403). We sincerely appreciate Ms Dandan Zhang for their excellent technical assistance.

References

1. Lull C, Wichers HJ, Savelkoul HF. Antiinflammatory and immunomodulating properties of fungal metabolites. Mediators Inflamm. 2005;2:63-80.
2. Ding ZH, Dong ZJ, Liu JK. Albaconol, a novel prenylated resorcinol (=Benzene-1,3-diol) from Basidiomycetes *Albatrellus confluens*. Helv Chim Acta. 2001;84:259-262.
3. Yang WM, Liu JK, Qing C, et al. Albaconol from mushroom *Albatrellus confluens* induced contraction and desensitization in Guinea pig trachea. Planta Med. 2003;69:715-719.
4. Qing C, Liu MH, Yang WM, Zhang YL, Wang L, Liu JK. Effects of Albaconol from the basidiomycete *Albatrellus confluens* on DNA topoisomerase II-mediated DNA cleavage and relaxation. Planta Med. 2004;70:792-796.
5. Sancho R, Lucena C, Macho A, et al. Immunosuppressive activity of capsaicinoids: capsiate derived from sweet peppers inhibits NF-κB activation and is a potent antiinflammatory compound *in vivo*. Eur J Immunol. 2002;32:1753-1763.
6. Liu Q, Shu X, Sun A, et al. Plant-derived small molecule albaconol suppresses LPS-triggered proinflammatory cytokine production and antigen presentation of dendritic cells by impairing NF-κB activation. Int Immunopharmacol. 2008;8: 1103-1111.
7. Liu Q, Chen T, Chen H, et al. Triptolide (PG-490) induces apoptosis of dendritic cells through sequential p38 MAP kinase phosphorylation and caspase 3 activation. Biochem Biophys Res Commun. 2004;319:980-986.
8. Liu Q, Chen T, Chen G, et al. Immunosuppressant triptolide inhibits dendritic cell-mediated chemoattraction of neutrophils and T cells through inhibiting Stat3 phosphorylation and NF-κB activation. Biochem Biophys Res Commun. 2006;345:1122-1130.
9. Zhang M, Tang H, Guo Z, et al. Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. Nat Immunol. 2004;5:1124-1133.
10. Wang Y, Chen T, Han C, et al. Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4. Blood. 2007;110:962-971.
11. An H, Hou J, Zhou J, et al. Phosphatase SHP-1 promotes TLR- and RIG-I-activated production of type I interferon by inhibiting kinase IRAK-1. Nat Immunol. 2008;9:542-550.
12. Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA. Possible new role for NF-κB in the resolution of inflammation. Nat Med. 2001;7:1291-1297.
13. Carmody RJ, Chen YH. Nuclear factor-κB: activation and regulation during toll-like receptor signaling. Cell Mol Immunol. 2007;4:31-41.
14. Zhu N, Cui J, Qiao C, et al. cAMP modulates macrophage development by suppressing M-CSF-induced MAPKs activation. Cell Mol Immunol. 2008;5:153-157.
15. Yasukawa H, Sasaki A, Yoshimura A. Negative regulation of cytokine signaling pathways. Annu Rev Immunol. 2000;18:143-164.
16. Crespo A, Filla MB, Russell SW, Murphy WJ. Indirect induction of suppressor of cytokine signalling-1 in macrophages stimulated with bacterial lipopolysaccharide: partial role of autocrine/paracrine interferon-α/β. Biochem J. 2000;349:99-104.
17. Nakagawa R, Naka T, Tsutsui H, et al. SOCS-1 participates in negative regulation of LPS responses. Immunity. 2002;17:677-687.
18. Kinjyo I, Hanada T, Inagaki-Ohara K, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity. 2002;17:583-591.
19. Beutler B. Innate immune response to microbial poisons: discovery and function of the Toll-like receptors. Annu Rev Pharmacol Toxicol. 2003;43:609-628.
20. Rice TW, Bernard GR. Therapeutic intervention and targets for sepsis. Annu Rev Med. 2005;56:225-248.

21. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol. 1997;15:323-350.
22. Payne DN. Nitric oxide in allergic airway inflammation. Curr Opin Allergy Clin Immunol. 2003;3:133-137.
23. Ali SD, Mann A. Signal transduction *via* the NF-κB pathway: a targeted treatment modality for infection, inflammation and repair. Cell Biochem Func. 2004;22:67-79.
24. Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. Nat Rev Drug Discov. 2004;3:401-416.
25. Cho JY, Baik KU, Jung JH, Park MH. *In vitro* anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*. Eur J Pharmacol. 2000;398:399-407.
26. Sheehan M, Wong HR, Hake PW, Zingarelli B. Protective effects of isohelenin, an inhibitor of nuclear factor κB, in endotoxic shock in rats. J Endotoxin Res. 2002;8:99-107.
27. Guardia T, Juarez AO, Guerreiro E, Guzman JA, Pelzer L. Anti-inflammatory activity and effect on gastric acid secretion of dehydroleucodine isolated from *Artemisia douglasiana*. J Ethnopharmacol. 2003;88:195-198.
28. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002;298:1911-1912.
29. Kimura A, Naka T, Muta T, et al. Suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production by regulating JAK-STAT. Proc Natl Acad Sci U S A. 2005;102: 17089-17094.