Protection of Lethal Toxicity of Endotoxin by *Salvia miltiorrhiza* BUNGE is via Reduction in Tumor Necrosis Factor Alpha Release and Liver Injury

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ABSTRACT

Lipopolysaccharide (LPS) has been implicated as one of the major cause of Gram-negative bacteria-induced sepsis that are life-threatening syndromes occurring in intensive care unit patients. Many natural products derived from medicinal plants may contain therapeutic values on protecting endotoxemia-induced sepsis by virtue their ability to modulate multiple pro-inflammatory cytokines. In the present study, we show that *Salvia Miltiorrhiza* (SM) BUNGE or Danshen, used in treatment of various systemic and surgical infections in the hospitals of China, was able to block the lethal toxicity of LPS in mice via suppression of TNF-α release and protection on liver injury. The ability of SM to suppress LPS-induced TNF-α release is further confirmed by *in vitro* experiments conducted on human peripheral blood leukocytes (PBL) and the RAW 264.7 macrophage cell line. Immunophenotyping by flow cytometry shows improved T-helper cell (CD4) and T-suppressor cells (CD8) ratio in SM-treated PBL and splenocytes of LPS-challenged mice. The drop in plasma glutamate-pyruvate transaminase (GPT) induced by LPS provides evidence that SM can protect hepatic damage. The present study explains some known biological activities of SM, and supports the clinical application of SM in the prevention of inflammatory diseases induced by Gram-negative bacteria.

Keywords: *Salvia miltiorrhiza*; Danshen; LPS; TNF

INTRODUCTION

Gram-negative bacteria-induced sepsis often contributed to the large percentage of deaths occurs in hospitalized patients [1]. Most of the toxic manifestations induced by Gram-negative bacteria are caused by lipopolysaccharide (LPS); an endotoxin derives from the cell wall of Gram-negative bacteria, which is the component of the outer membranes of these organisms. The pathological manifestations attributed to LPS include circulatory shock, disseminated intravascular coagulation, and failure of numerous organs, including the central nervous system, heart, kidneys, gastrointestinal tract, lungs and liver [2-5]. Excessive release of proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), gamma interferon (IFN-γ) from the stimulated monocytes and tissue macrophages is said to be responsible for many of the pathological responses induced by LPS [4,6,7].

Antibiotic therapy does not prevent the toxic manifestations of LPS, and may even promote the release of LPS from bacteria [8,9]. Several clinical trails conducted with antagonists to TNF-α [10] failed to produce significant therapeutic benefits for septic patients partly because sepsis is not the result of the release of any one cytokines. Recent studies show that some natural products derived from medicinal plants may contain therapeutic values on protecting endoxemia-induced sepsis due to their ability to modulate multiple proinflammatory cytokines. Among the medicinal plants, anti-inflammatory substances derived cinnamon bark [11], biscoclaurine alkaloid cepharanthin [12] and naringin [13] have all been shown to be capable of blocking TNF-α release and the lethal shock induced by LPS. In attempt to search for natural components with protective properties of gram-negative bacterial infection, we have demonstrated that *Salvia miltiorrhiza* (SM) is also capable of suppressing the endotoxemia induced by LPS via suppression of TNF-α release and liver protection.

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Salvia miltiorrhiza BUNGE or Danshen is officially listed in the Chinese Pharmacopenia and is best known for its unique application in the treatment of coronary heart diseases, hemorrhages, menstrual disorders and miscarriages, renal diseases, myocardial infarction and hypertension [14-17]. The protection of SM has been associated with its effects on cerebral edema, monoamines, neuropeptides, and neurotransmitter amino acids, thromboxane A2, peroxidation and downregulation of c-fos gene expression [18]. The plant is a rich source of polyphenols, with an excess of 160 polyphenols has been identified [19,20]. The active constituents of SM can be classified into two groups. The first group consists of the phenolic compounds slavianolic acid and lithospermate B; and the second group consists of the abietane type-diterpene quinone pigments tanshinone I, tanshinone IIA, tanshinone IIB and the cryptotanshinone [19-21], rosmarinic acid and lithospermic acid [22]. The therapeutic effect of SM on heat stroke, myocardial ischemia and infarction has been largely attributed to the endothelium-dependent vasodilatory and hypertensive properties of the tanshinones [19-21] and lithospermic acid B [23]. The salvianoic acids A, B, and C of SM have also been shown to capable of protecting liver microsomes, hepatocytes and erythrocytes against oxidative damage [22] and recently showed to protect human neuroblastoma cells against cytotoxicity. Rosmarinic acid is a naturally occurring polyphenol with anti-oxidative and anti-inflammatory activities [25-27] and its salts are reported to have anti-HIV activities [28].

SM or Danshen is frequently used as a decoction or intravenous injection in the treatment of various systemic and surgical infections in the hospitals of China. Successful outcomes have been observed in treating infections of hands and feet, erysipelas, ostemyelitis, matitis, cellulitis, otitis externa acute tonsillitis as well as infections of bone and joints but the anti-infection mechanistic actions have not been investigated. The present study sought to investigate the protective mechanisms of SM

on gram. By using an *in vivo* LPS-challenged mouse model, we have demonstrated that SM exhibits ant-inflammatory properties and suppressed the pro-inflammatory TNF-α cytokine release and liver damage induced by LPS in mice. The ability of SM to suppress LPS-induced pro-inflammatory TNF-α cytokine is further supported by the two separate *in vitro* experiments conducted with human peripheral blood leukocytes and macrophage RAW 264.7 cell line. The present study thus helps to explain some known therapeutic properties of SM.

MATERIALS AND METHODS

Reagents

The trade name of *Salvia miltiorrhiza* (SM) is H-care (batch no. 3HC1980808, supplied by Winsor Health Products Ltd., Hong Kong). Reagents for cell culture, Trizol^{TM}, and PCR primers were purchased from Invitrogen (Carlsbad, Ca). Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO). ELISA kit for mouse TNF-α was purchased from Endogen (Woburn, MA). Anti-CD16/CD32, anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-phycoerythrin (PE), anti-CD8-PE antibodies, TMB substrate solution, ELISA kits for human TNF-α were supplied by PharMingen (San Diego, Ca). Buffy coats were obtained from Hong Kong Red Cross Blood Transfusion Service. Microplate reader was supplied by BioRad. The stock solution of *Salvia miltiorrhiza* was prepared by dissolving the dry SM capsule powder (10 mg/ml) in distilled water on a rolling plate for 30 minutes. The solution was centrifuged at 4,000 g for 20 minutes and the supernatant was further freeze-dried by Heto DryWinner (Denmark). The aqueous soluble fraction was collected after passing through a sterilized 0.22 μm filter.

Mouse-treatment of SM and LPS

Male BALB/c mice aged 6-8 wk were obtained from the animal house of the department of Zoology, the University of Hong Kong. All mice were kept at 22°C and 55% relative humidity in a 12-hour day/night rhythm with free access to laboratory chow and water. The mice were randomly divided into 2 groups of 12 animals and designated to receive orally either 0.3 ml of deionized water or SM (125 mg/kg/day) solution for a period of 38 days. The body weight of the animals was recorded every two days. After 38 days of oral feeding, half of the animals from each group received an

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intraperitoneal injection of lipopolysaccharide (LPS) (20 mg/kg). The other half received 0.9% saline and was served as Control. All experimental procedures were performed under license issued by the Government of Hong Kong SAR and endorsed by the Committee on the use of Live Animals in Teaching and Research of the University of Hong Kong.

Biochemical analysis of blood samples in mouse

After 24 hours of LPS injection, all mice were anesthetized with carbon dioxide and killed by exsanguination via heart puncture. Blood were collected from the heart by using a 25-gauage syringe. The plasma was stored at 4°C until further analysis of TNF-α, lymphocyte subsets immunophenotyping and glutamate-pyurate transaminase (GPT) activity by ELISA, flow cytometry and spectrophotometry, respectively.

Preparation of mouse peripheral blood leukocytes (PBL) and splenocytes

Mouse peripheral blood leukocytes were isolated by using the red blood cell lysing buffer. 100 μl of blood sample was diluted (1:20) with red blood cell lysing buffer and placed at room temperature for 3 minutes. The mixture was centrifuged at 400 g for 5 minutes and the supernatant was aspirated. The pellet contained the PBL was washed with phosphate buffer solution (PBS) and resuspended in 50 μl blocking buffer (PBS, 1% BSA and 0.1% sodium azide) on ice.

Splenocytes were prepared by dissociate the spleen in the cell strainer using a syringe plunger. The exudates were centrifuged at 400 g for 5 minutes and the red blood cells were removed by adding 2 ml of red blood cell lysing buffer. After washing with PBS, the splenocytes (1 x 10^6 cells) were resuspended in 50 μl blocking buffer on ice.

Immunophenotyping of mouse peripheral blood leukocytes and splenocytes by flow cytometry

Mouse PBL and splenocytes were pre-incubated with 0.5 µg of anti-CD16/CD32 antibody for 15 minutes on ice to block the Fc receptors. Their lymphocyte subsets (total-T (CD3), T-helper/inducer (CD4) and T-cytotoxic/suppressor (CD8)) were immunophenotyped with monoclonal antibodies conjugated with fluorochromes. The anti-CD3 (total T subset) antibody was conjugated with fluorescein isothiocyanate (FITC); the other two antibodies (CD4 and CD8) were conjugated with phycoerythrin (PE). Briefly, 20 µl of monoclonal antibody was added to the cell suspension and the mixture was incubated in the dark for 30 minutes. Before flow cytometric analysis, 400 µl of blocking buffer was added to the mixture.

Determination of glutamate-pyurate transaminase (GPT) activity in mouse serum

To determine glutamate-pyurate transaminase (GPT) activity in serum, 100 µl of serum was mixed with 500 µl of preincubated (37°C) GPT substrate solution (1.8 mM α-ketoglutarate and 200 mM DL-alanine in 0.1 M phosphate buffer, pH 7.2) in test tube. The mixture was then incubated in a water bath at 37°C for 30 minutes. 500 µl of 1 mM 2,4-dinitrophenylhydrazine (DNP) was added and the mixture was placed at room temperature for 20 minutes. Finally, 5 ml of 0.4 M NaOH was added and kept at room temperature for 5 minutes for color development. Absorbance of the mixture was measured at 505 nm.

The *in vitro* treatment of LPS and SM on human peripheral blood leukocytes (PBL)

Fresh human PBLs were isolated buffy coats obtained from healthy blood donors. Briefly, PBLs were prepared by using red blood cell lysing buffer (NH_4Cl 0.83%, $NaHCO_3$ 0.084%, EDTA 0.003%). The blood was diluted (1:45) with red blood cell lysing buffer and placed at room temperature for 3 minutes. Then the mixture was centrifuged at 400 g for 5 minutes. The supernatant was decanted and the cell pellet was resuspended and washed twice in complete RPMI 1640 medium. The cells were counted by Coulter Multisizer and seeded at a cell density of 2×10^5 cells per well in 96 well tissue culture plates. The cells were treated with or without LPS (10 μg/ml) and SM (0, 100 and 400 μg/ml) for 72 hours. Supernatant was collected at 72 hours for TNF-α detection by ELISA.

In vitro treatment of Raw 264.7 macrophage cell line with LPS and SM

The Raw 264.7 macrophage cell line (originally from the American Type Culture Collection) was kindly donated by Dr. R.C.C. Chang (The University of Hong Kong, HKSAR, China). The cells were grown in $25cm^2$ flasks in DMEM containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin and 1% fungizone at 37°C in 5% CO_2 incubator. For all experiments, cells were grown to ~80% confluence and were subjected to no more than 25 cell passages. The cells (5×10^6 cells/$25cm^2$ flasks) were pre-incubated with SM (0, 100 and 400 μg/ml) for 16 hours before exposed to LPS (1 μg/ml). Supernatant and cells were collected 3 hours after exposure to LPS for the detection of TNF-α by ELISA and RT-PCR.

ELISA assay for TNF-α measurement

Commercial ELISA kits for mouse/human TNF-α were used. The cytokine production was analyzed by sandwich ELISA. Briefly, ELISA plates were coated with appropriate capture antibodies diluted in sodium bicarbonate buffer (0.1M, pH 9.5)

overnight at 4°C. Plates were washed five times with washing buffer (0.05% Tween-20 in PBS) between steps. The wells were blocked with 200 μl of assay diluents (10% FBS in PBS) for 3 hours at room temperature and 100 μl of supernatant or serum were added and incubated for 2 hours. Next, 100 μl of the working detector (detection antibody and avidin-horseradish peroxidase conjugate) was added and incubated for 1 hour at room temperature. Finally, color development was achieved by enzymatic reaction brought by incubation with 100 μl TMB substrate solution for 30 minutes and the reaction was terminated by adding 50 μl of 2 M sulphuric acid. The absorbance was measured on a microplate reader at 450 nm.

Analysis of TNF-α mRNA levels in Raw 264.7 macrophage cell line by RT-PCR

Total RNA was isolated from Raw264.7 cells according to the instructions of the manufacturer. Briefly, cells were lysed by adding 2 ml of Trizol™ to the flask. Total RNA concentration was measured by spectrophotometry (absorbance at 260nm and 280nm). The RNA preparation was controlled by minigel agarose electrophoresis with visualization of the 18S and 28S ribosomal RNA bands after ethidium bromide staining. One μg of total RNA from each sample was reverse transcribed in 20 μl of a reaction buffer containing reverse transcriptase from murine leukemia moloney virus (M-MLV RT). For PCR, 1 μl of cDNA was amplified by PCR by using the following specific primers:

Gene	Primer sequences (5'-------> 3')
TNF-α	GGC CTT CCT ACC TTC AGA CC
	AGC AAA AGA GGA GGC AAC AA
GAPDH	GGT GAA GGT CGG TGT GAA CG
	GGT AGG AAC ACG AAA GGC CA

The reaction mixtures of TNF-α and GAPDH were subjected to 30 cycles and 25

cycles denaturation (95°C, 3 min) respectively, annealing for 30 s at 60°C and extension for 30 s at 72°C. A final extension was 10 min at 72°C. The amplicons were visualized on a 1.5% ethidium bromide stained agarose gel. Intensities of band were compared after visualization on an UV transilluminator and analyzed by Image Master Total Lab (Amersham pharmacia biotech).

Statistical analysis

Statistical significance of the data was determined by one-way analysis of variances (ANOVA). Results were compared by Student's paired t test with a two-tailed probability value $p< 0.05$ taken as significant.

RESULT

Protection of SM on LPS-induced body weight change

Figure 1 shows that chroming feeding of SM did not affect the normal growth pattern of the animals throughout the feeding period. Thirty minutes post LPS injection, sign of reduced physical activities as well as shock symptoms such as bloody nose, anorexia and diarrhea were observed in all the animals. These classical pathological symptoms of LPS were however less apparent in animals with SM treatment. The protective effect on LPS-induced toxicity is supported by the significant ($p < 0.05$) protection on the reduction of body weight caused by LPS in the SM-treated mice as compared to the non-SM-treated animals.

Suppression of SM on LPS-induced TNF-α level measured in mouse plasma, human peripheral blood leukocytes culture and Raw 264.7 macrophages.

Since excessive release of the pro-inflammatory cytokine TNF-α is often associated with many of the pathological responses induced by LPS [7], we have detected its serum level 24 hours after LPS injection in the animals. Figure 2 showed that the basal circulatory level of TNF-α in the control (non-LPS) mice was not affected by SM. Upon LPS challenge, the plasma level of TNF-α was enhanced by three-fold. LPS-induced elevation serum TNF-α in mice was significantly ($p<0.05$) reduced from 146 pg/ml to 85 pg/ml with SM treatment.

The ability of SM to reduce TNF-α release was further tested by carried out two independent *in vitro* experiments using different cells of the immune response. Figure 3 shows that the secretion of TNF-α was reduced ($p < 0.01$) by SM dose-dependently in both Control and LPS treated human peripheral blood leukocytes. At 100 µg/ml of SM,

the level of TNF-α reduced by 30% and 35% in the Control and LPS treated PBL, respectively.

The ability of SM to suppress TNF-α is further confirmed by the Raw264.7 macrophages cell line. Figure 4A shows that only small amounts of TNF-α were detectable by the ELISA assay in the supernatant of the non-LPS treated Raw 264.7 cells. Upon LPS challenge, the TNF-α level of the cultured macrophages cells (without SM treatment) was enhanced by three hundred-fold. SM at 100 μg/ml and 400 μg/ml dose level significantly suppressed the TNF-α release by 15% and 30%, respectively. Figure 4B presented the RT-PCR data indicating SM inhibits LPS-induced TNF-α release likely at the mRNA level.

Elevation of CD4/CD8 ratio in spleen and peripheral blood mononuclear cells of LPS-treated mice by SM

Challenging the BALB/c mice with LPS derived from Escherichia allowed us to assess both humoral and cell-mediated immune resistance. Immunophenotying by flow cytometry of both peripheral blood T-lymphocytes and splenocytes clearly illustrated that mice receiving SM treatment manifested a lower degree of injury upon LPS challenge. Following LPS challenge, the absolute lymphopenia of CD3+ lymphocytes was observed. Upon stimulation of LPS, there is usually a massive decline in CD4+ T helper cells with the CD8+ T cytotoxic cells either remain unchanged or elevated (Ertel and Faist, 1989). This leads to a conversion of the CD4/CD8 ratio to decrease dramatically. In this study, we found that LPS induced similar changes in both T-lymphocytes (Table 1) and splenocytes (Table 2) as shown by a decrease in CD4+ and CD8+ cells accompanied by a decrease in the CD4/CD8 ratio.

Suppression of LPS-induced GPT Level by SM

Plasma glutamate-pyruvate transaminase (GPT) level was used as an index of hepatotoxicity. Figure 5 shows that the plasma GPT level of the non-SM (Control) and SM fed healthy mice (SM) was similar. LPS induced an 8-fold increase of the liver GPT level in the non-SM treated mice and this elevation was significantly lowered with SM treatment. The liver LPS-induced GPT elevation level was significantly lower in the SM treated mice (156.32 ± 1.45 unit) as compared to the non SM treated mice (386.32 ± 2.14 unit).

DISCUSSION

Endotoxin has been recognized as one of the major causes of Gram-negative bacteria-induced sepsis that are life-threatening syndromes occurring often in intensive care unit patients [3,29]. The pro-inflammatory cytokines tumor necrosis factor-α and interleukin–1α have been implicated as principal mediators during endotoxemia [6,30]. Infusion of high doses of these mediators results in severe tissue damage, organ failure and death [6,7]. TNF-α is regarded as the main executor of many deleterious effects of endotoxaemia and an important marker of severity in sepsis [29] and dysfunction [4,6,30].

Recently, *Salvia miltiorrhiza* BUNGE or Danshen attracts many scientists because of its many tanshinones and phenolic compounds exhibit diverse biological activities such as anti-inflammatory, anti-bacterial, antitumor, antioxidant, and anti-mutagenesis [16,29,31-35]. By using the LPS-induced endotoexomia animal model, this is the first report to reveal that the anti-inflammatory properties of SM involves suppression of the pro-inflammatory cytokine tumor necrosis factor alpha, improvement of T-cell immunity and protection of liver injury. The ability of SM to suppress LPS-induced TNF-α release is further supported by its dose-dependent lowering effect observed with the isolated human peripheral blood leukocytes (Figure 3) and Raw264.1 macrophages cell line (Figure 4) *in vitro*. To what extents the control of TNF-α release by SM contributed to the execution of the many deleterious effects of endotoxaemia observed in this study has yet to be determined. The inhibition on pro-inflammatory cytokine TNF-α activity, however, has been regarded as an important anti-atherosclerotic property of SM in treatment of acute coronary syndromes [36]. Both aqueous ethanolic extract (SME) and the water-soluble antioxidant, savianolic acid B (Sal B) of SM, have

been shown to inhibit TNF-α induction on nuclear factor kappa B (NF-κB) expression in human aortic endothelial cells (HAECs) line [37].

Immunophenotyping by flow cytometry of both peripheral blood leukocytes and splenocytes in mice illustrated for the first time that SM can play an important role in cell-mediated immune resistance. Following endotoxin challenge, the absolute lymphopenia of CD3+ lymphocytes was observed. In both splenocytes and T-lymphocytes, the helper cell (CD4+) and suppressor cell (CD8+) populations were greatly reduced after LPS treatment with the CD4+ population decreased in a greater extent than CD8+. The absolute percentages of lymphocyte subsets from spleen and blood were slightly different and their response to SM treatment was not identical. On the whole, SM improved the CD4+/CD8+ ratio; and this ratio of lymphocytes in the blood were more sensitive to both LPS and SM than that of the spleen.

The mechanism(s) by which SM protects LPS-induced deletion of the T-helper cells (CD4) observed in this study is unknown. Deletion of T-lymphocytes is often resulted from programmed cell death caused by several adverse conditions such as ligation of Fas/APO-1/CD95, CD30, TNF receptor, and cytotoxic T lymphocyte antigen on T cells [38]. On one hand, the lymphocytes require to receive the corresponding signals to mount a productive immune response such as to produce cytotoxic T-cells (CD8) to destroy antigen-bearing target or secrete soluble cytokines. On the other hand, failure to provide the proper signals could either abort or imbalance such immune response. Among the many active components, both rosmarinic acid and tanshinones of SM are likely candidates. It has been suggested that rosmarinic acid can affect the mediation of T-cells through suppression on the inflammatory eicosanoids and free radicals, both of which have been showed to inhibit lymphocyte proliferation [39,40]. At concentrations of 10^{-5}-10^{-3}M [26], rosmarinic acid has been shown to inhibit the formation of the inflammatory eicosanoids such as the 5-hyroxy-6, 8, 11, 14-eicosatetraenoic acid

(5-HETE) and leukotriene B4 (LTB4) (5-lipooxygenase products). Rosmarinic acid has been considered as a new inhibitor of complement C3-convertase with anti-inflammatory activity [25]. It can inhibit cytokine-induced proliferation of murine mesangial cells and suppress c-myc mRNA expression in PDGF-stimulated mesangial cells [27]. The tanshinones and crypotanshinone of SM, on the other hand, can favor the T-cell specific immune defense of the mice either by promoting differentiation or rescuing deletion of the T-helper cells (CD4), or both, via suppression of LPS-induced nitric oxide (NO) production. NO is synthesized via the oxidation of arginine, by a family of nitric acid synthases (NOS), and plays a vital role in apoptotic cell death pathways of LPS. Several tanshinones and crypotanshinone have been shown to be capable of reducing LPS-induced NO production and interfering with the expression of the molecular target genes NOS, NF-κB, and ERK that are involved in the apoptotic signaling pathway of LPS in the Raw 264.7 macrophages [41,42].

The glutamate-pyruvate transaminase (GTP) is a cytosolic enzyme of the liver cells. The increase plasma level of GTP is often associated with hepatic necrosis [43-45] and the cell death process is accompanied by plasma membrane permeability increase [46-49]. Oral feeding of SM had no adverse effect on the plasma glutamate-pyruvate transaminase of the healthy animals. LPS challenge significantly elevated a 4-fold increase of the GPT activity as compared to the control. SM reduced LPS-induced GPT elevation and a 275 SF unit/ml detected difference was observed between the SM-treated and non SM-treated mice. The reduction of GPT by SM indicates its protective potential on liver injury. In recent finding, it has been shown that SM is capable of protecting liver fibrosis via its ability to suppress many of the fibrosis-related genes including the α-smooth muscle growth factor, connective tissue growth factor, and tissue inhibitor of metalloproteinase-1 [35]. The protection of lipopolysaccharide-induced hepatic damage has been demonstrated by other medicinal plant components including *edaravone* [43],

gentiopicroside [50], *Hibiscus protocatechuic* acid [51]. Their common mechanisms are suppression of the inflammatory cytokine and reduction in necrosis of the liver caused by the oxidative stress. Whether SM protected LPS-induced liver damage was via its scavenging effect on superoxide and inhibition on TNF-α release remains to be determined.

In conclusion, the information provided in the current study helps to explain some of the clinical benefits of *Salvia miltiorrhiza* (commercial name H-care) observed during treatments of bacterial infection in hospitals. The anti-inflammatory mechanism of actions of SM includes modifying of both humoral immunity and non-specific immunity as well as alleviating hepatotoxicity. Further studies are, however, warranted to identify the individual active compounds that are responsible for the immunoregulatory activities of *Salvia miltiorrhiza* BUNGE.

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Legend

FIG. 1. Effect of *Salvia Miltiorrhiza* on mouse body weight. Male BALB/c mice were orally fed with or without *Salvia Miltiorrhiza* (SM) (125 mg/kg/day) for a total of 39 days. On day-38, half of the animals received intraperitoneal injection of LPS (20 mg/kg) and the other half received 0.9% saline. Data are mean $\pm$ SEM for twelve and six mice per group before and after day 39, respectively. * $p<0.05$ vs Control+LPS.

FIG. 2. Effect of *Salvia Miltiorrhiza* on TNF-α expression in mouse peripheral blood leukocytes. Male BALB/c mice were orally fed with or without *Salvia Miltiorrhiza* (SM) (125 mg/kg/day) for 39 days. On day 38, half of the animals received intraperitoneal injection of LPS (20 mg/kg) and the other half received 0.9% saline. Peripheral blood leukocytes were isolated and their expression of TNF-α was determined by ELISA. Data are mean $\pm$ SEM for six mice/group. * $p<0.001$ as compared with Control group (no SM).

FIG. 3. Effect of *Salvia Miltiorrhiza* on TNF-α expression in human peripheral blood leukocytes. Human peripheral blood leukocytes were treated with or without LPS (10 μg/ml) and *Salvia Miltiorrhiza* (SM) (0, 100 and 400 μg/ml) for up to 72 hours. Supernatants were collected and TNF-α expression was determined by ELISA. Data are mean $\pm$ SEM (n=12). *, ** $p<0.05$, 0.01, respectively as compared with 0 μg/ml of SM treatment.

FIG. 4. Effect of *Salvia Miltiorrhiza* on TNF-α expression in Raw 264.7 macrophages. Raw 264.7 macrophages were pre-incubated in the presence of *Salvia Miltiorrhiza* (SM) (0, 100 and 400 μg/ml) for 16 hours and subsequently treated with or

without LPS (1 μg/ml). Cells and supernatants were collected at 3 hours after exposure to LPS. (A) TNF-α expression was determined by ELISA. Data are mean ± SEM (n=6). *, *** $p<0.05$, 0.001, respectively as compared with 0 μg/ml of SM treatment. (B) TNF-α expression was determined by RT-PCR and the amount of RNA loaded in each lane was confirmed by GAPDH mRNA.

FIG. 5. Effect of *Salvia Miltiorrhiza* on mouse plasma level of glutamate-pyruvate transaminase. Male BALB/c mice were orally fed with or without *Salvia Miltiorrhiza* (SM) (125 mg/kg/day) for 39 days. On day 38, half of the animals received intraperitoneal injection of LPS (20 mg/kg) and the other half received 0.9% saline. Glutamate-pyruvate transaminase (GPT) level in serum was analyzed as mentioned in Materials and Methods. Data are mean ± SEM for six mice/group. * $p< 0.001$ as compared with non-LPS group.

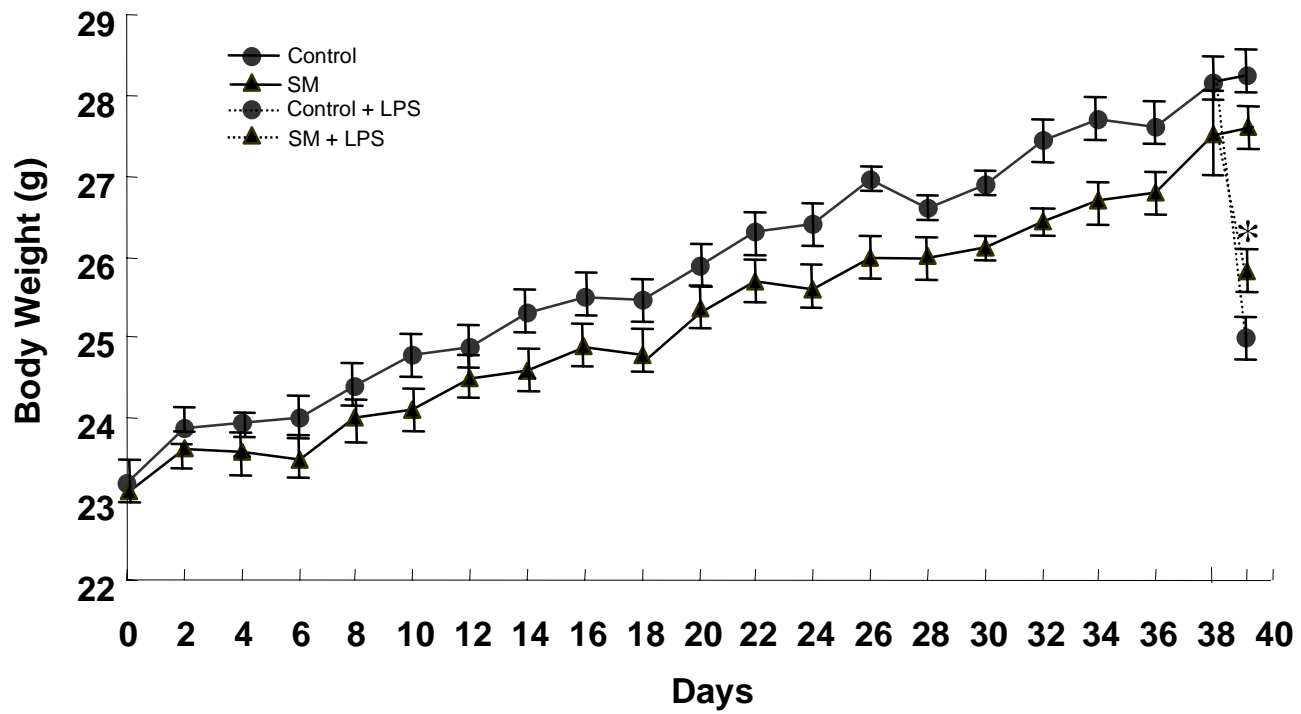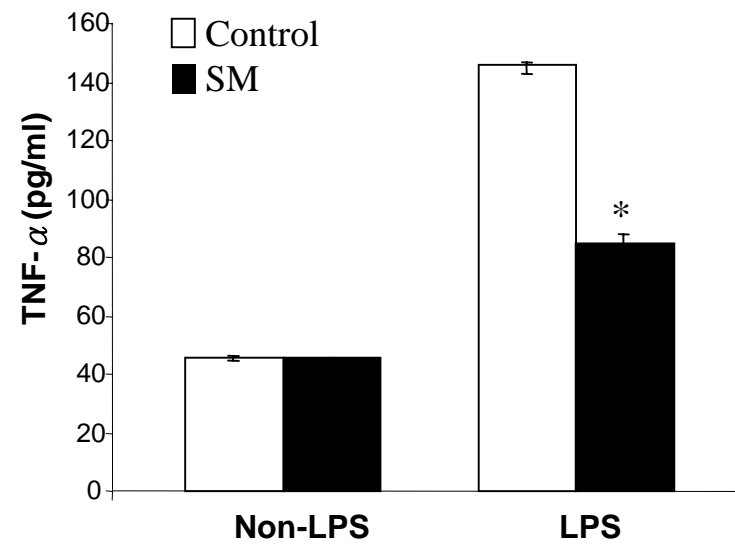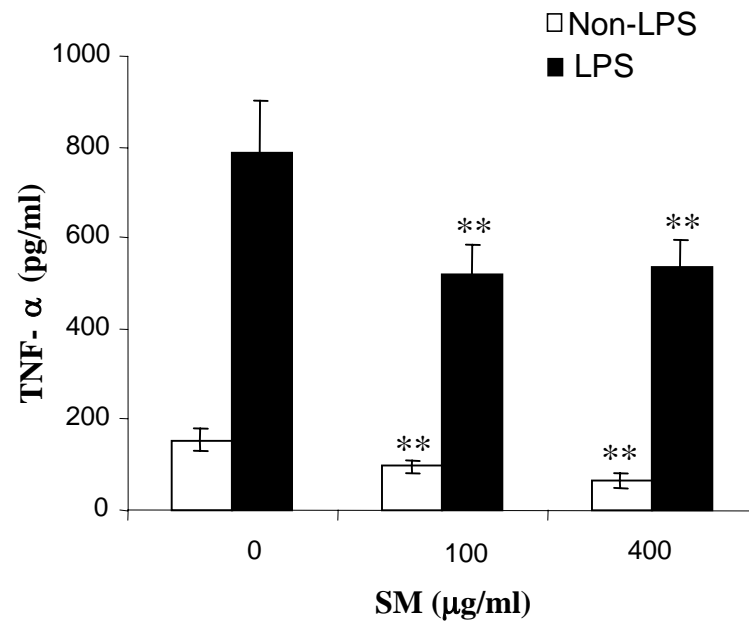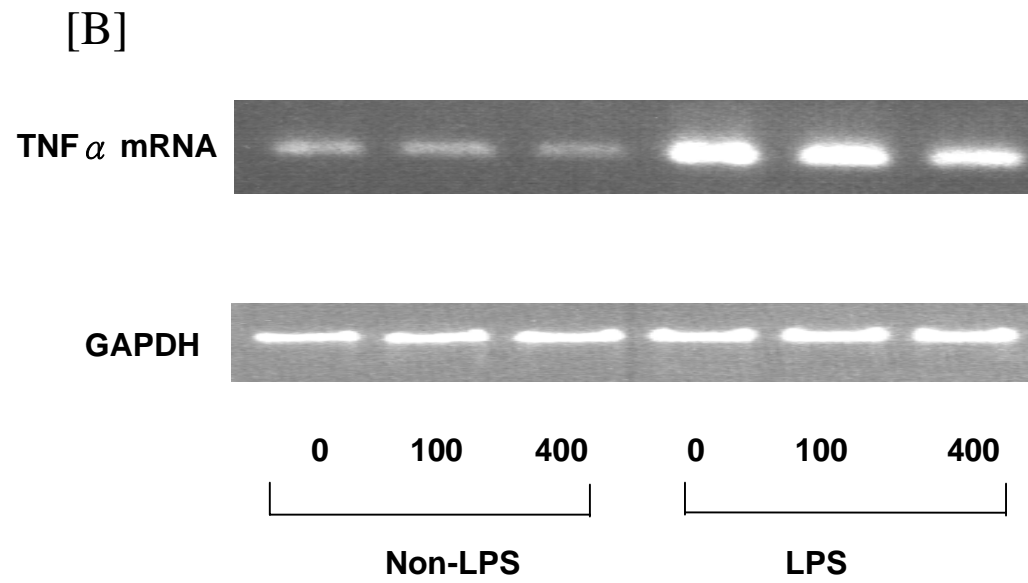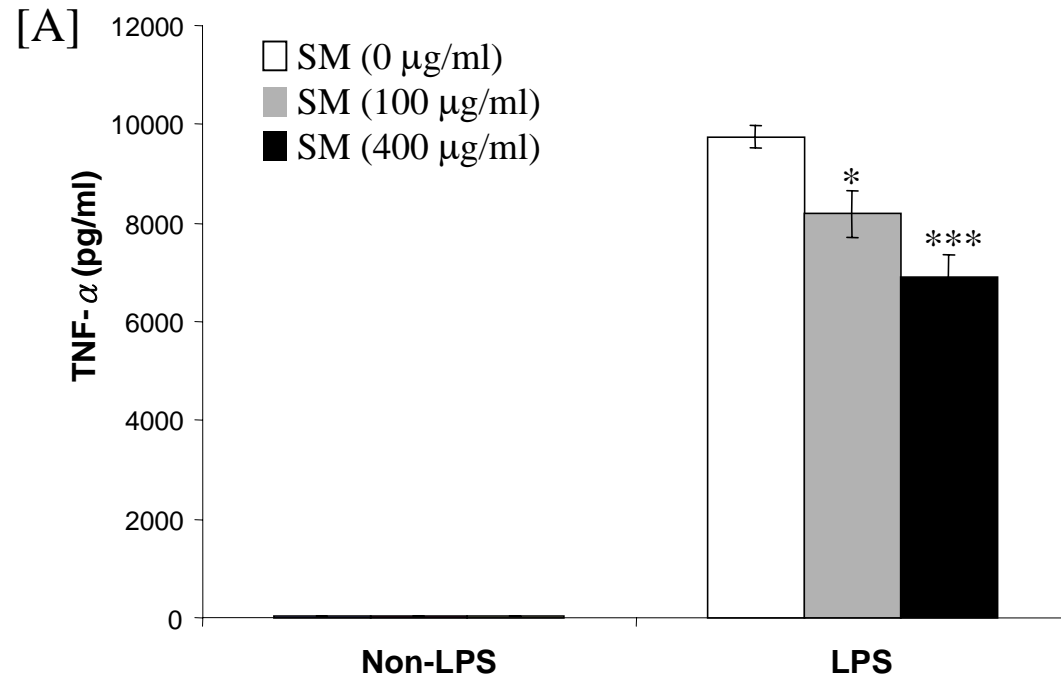Fig. 1

Fig. 2

Fig. 3

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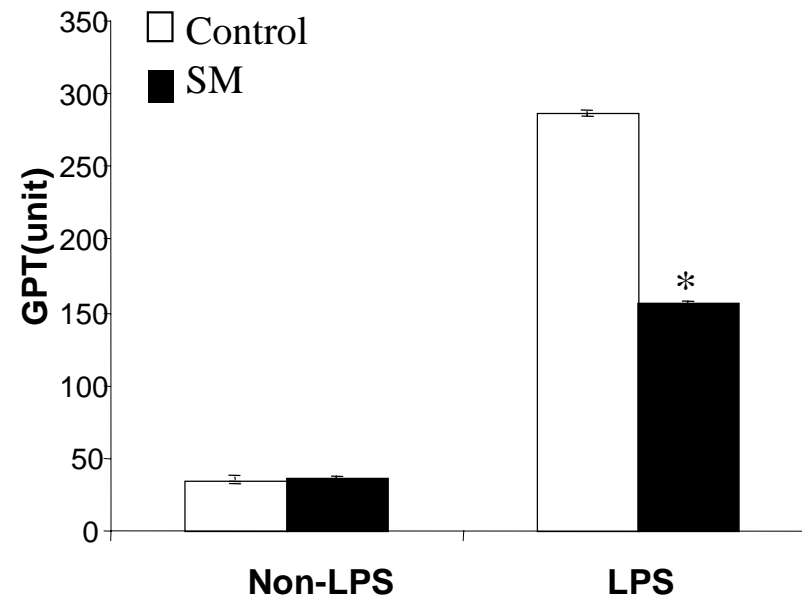
Fig. 5

Table 1. Effects of *Salvia miltiorrhiza* on the mouse T-lymphocytes subpopulation challenged by LPS

Treatment	Non-LPS			LPS		
	% Lymphocyte subset			% Lymphocyte subset		
	CD8	CD4	CD4/CD8	CD8	CD4	CD4/CD8
Control	17.38 ± 0.71	41.14 ± 3.03	2.36 ± 0.09	5.74 ± 0.19	10.84 ± 0.45	1.89 ± 0.04
SM	16.63 ± 0.60	46.49 ± 1.38	2.80 ± 0.03	5.78 ± 0.27	13.95 ± 0.55	2.43 ± 0.14*

Male BALB/c mice were orally fed with or without *Salvia Miltiorrhiza* (SM) (125 mg/kg/day) for 40 days. On day 38, half of the animals received intraperitoneal injection of LPS (20 mg/kg) and the other half received 0.9% saline. Mouse peripheral blood leukocytes were isolated as described in Materials and Methods. Immunophenotype determination was carried out by flow cytometry.

Data are mean ± SEM for six mice/group.

*p<0.05 vs Control group

Table 2. Effects of _Salvia miltiorrhiza_ the mouse Splenocytes subpopulation challenged by LPS

Treatment	Non-LPS			LPS		
	% Lymphocyte subset			**% Lymphocyte subset**		
	CD8	**CD4**	**CD4/CD8**	**CD8**	**CD4**	**CD4/CD8**
Control	16.47 ± 0.70	33.54 ± 1.18	2.03 ± 0.02	19.63 ± 1.12	25.16 ± 0.78	1.29 ± 0.02
SM	17.72 ± 1.35	33.72 ± 2.05	1.97 ± 0.04	17.45 ± 0.50	30.02 ± 0.67	1.72 ± 0.03***

Male BALB/c mice were orally fed with or without _Salvia Miltiorrhiza_ (SM) (125 mg/kg/day) for 40 days.
On day 38, half of the animals received intraperitoneal injection of LPS (20 mg/kg) and the other half
received 0.9% saline. Mouse splenocytes were isolated as described in Materials and Methods.
Immunophenotype determination was carried out by flow cytometry.
Data are mean $\pm$ SEM for six mice/group.
***$p < 0.001$ vs Control group