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# Inhibition of Lipogenesis Reduces Inflammation and Organ Injury in Sepsis

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## Abstract

**Background**—Sepsis is a life threatening acute inflammatory condition associated with metabolic complications. Accumulation of free fatty acids (FAA) induces inflammation and causes lipotoxic effects in the liver. Since fatty acid metabolism plays a role in the inflammatory response, we hypothesized that the administration of C75, a fatty acid synthase inhibitor, could alleviate the injury caused by sepsis.

**Methods**—Male mice were subjected to sepsis by cecal ligation and puncture (CLP). At 4 h after CLP, different doses of C75 (1 or 5 mg/kg BW) or vehicle (20% DMSO in saline) were injected intraperitoneally. Blood and liver tissues were collected at 24 h after CLP.

**Results**—C75 treatment with 1 mg- and 5 mg/kg BW significantly lowered FFA levels in the liver after CLP by 28% and 53%, respectively. Administration of C75 dose dependently reduced serum indexes of organ injury (AST, ALT, LDH) and serum levels of TNF-α and IL-6. In the liver, C75 treatment reduced inflammation (TNF-α, IL-6) and oxidative stress (iNOS, COX-2) in a dose-dependent manner. The 5 mg dose improved the 10-day survival rate to 85% from that of 55% in the vehicle. In the presence of C75, TNF-α release in RAW 246.7 cells with 4 h LPS stimulation were also significantly reduced.

**Conclusions**—C75 effectively lowered FFA accumulation in the liver, which was associated with inhibition of inflammation and organ injury as well as improvement in survival rate after CLP. Thus, inhibition of FFA by C75 could ameliorate the hepatic dysfunction seen in sepsis.

## Keywords

C75; sepsis; inflammation; injury; free fatty acid; lipogenesis

## INTRODUCTION

Sepsis is a frequently critical condition characterized by an amplified and widespread inflammatory response. Failure to expunge invading pathogens from the body leads to disordered inflammation, overproduction of inflammatory cytokines, multiple organ dysfunction, and death. It accounts for over 10% of intensive care unit fatalities and is the third most common cause of death in the United States, Australia, and Asia. Mortality rates are higher than the four most deadly forms of cancer combined [1–3]. Despite the advances in the understanding of sepsis pathology over the last several decades, no therapy other than supportive care is available for sepsis patients. Activated protein C, the only drug approved by the FDA as therapy for sepsis has recently been withdrawn due to adverse reactions. Supportive care such as broad spectrum antibiotics, fluid administration and oxygen delivery are still the course of treatment for sepsis patients. Numerous agents that showed promise in preclinical studies as therapy for sepsis have failed in clinical trials [2,4–9]. Better understanding of the pathophysiology of sepsis is imperative in developing effective therapeutics.

C75 is a synthetic cell permeable small molecule inhibitor of fatty acid synthase (FAS) which inhibits long-chain fatty acid elongation [10,11]. C75 interacts with two targets in the lipid metabolism pathway: one is the FAS and the other is the carnitine palmitoyl transferase 1 (CPT-1). FAS is the primary enzyme responsible for *de novo* synthesis of fatty acids which catalyze the condensation of malonyl-CoA and acetyl-CoA to produce palmitate. CPT-1 is the rate limiting enzyme required for mitochondrial fatty acid oxidation and subsequent energy production. Malonyl-CoA is both the substrate of FAS and an allosteric inhibitor of CPT-1 which prevents the oxidation of newly synthesized fatty acids formed during lipogenesis. C75 inhibits FAS and increases malonyl-CoA. However, C75 stimulates CPT-1 even in the presence of high levels of malonyl-CoA [12]. Thus C75 is credited for both inhibiting lipogenesis and stimulating mitochondrial fatty acid oxidation [11].

Clinical studies have established a link between lipid metabolism and systemic inflammation [13–15]. Currently, it is not clear whether targeting fatty acid metabolism could attenuate the severity of sepsis. Alterations in plasma fatty acid profile are seen in patients with septic shock and such profile can be attributed to the activation of hepatic *de novo* lipogenesis. This leads to hepatosteatosis, an increase in adipose tissue lipolysis, the increased fatty acid-induced oxidative stress and increased production of inflammatory lipid mediators [16–18]. Therefore, we hypothesized that C75 could serve as an inhibitor of lipogenesis thereby attenuating inflammation and organ injury associated with sepsis.

## MATERIALS AND METHODS

### Cecal ligation and puncture (CLP) model

Male C57BL/6 mice (20–25 g; Taconic, Albany, NY) were used for the study and inhalable isofluorane was administered as an anesthetic. A midline laparotomy incision was performed to expose the abdomen and cecum. The cecum was ligated at the ileo-cecal valve and punctured twice with a 22 gauge needle. The abdominal incision was closed in layers

and mice were allowed to recover from anesthesia. At 4 h after CLP, mice were administered 1 mg/kg BW (1 mg dose), 5 mg/kg BW (5 mg dose) of C75 (4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid; Sigma-Aldrich, St. Louis, MO) or vehicle (20% DMSO in normal saline) intraperitoneally. Sham animals underwent a midline laparotomy incision and closure, without CLP or treatment. At 24 h, blood and liver tissues were collected and frozen immediately in liquid nitrogen, and stored at −80°C until analysis. All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health (Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research.

### Survival assessment

Sepsis was induced by CLP as described above. Immediately after CLP, an antibiotic (i.e., imipenem) at a dose of 0.5 mg/kg BW was given once subcutaneously. At 4 h after CLP, mice were administered 5 mg dose of C75 or vehicle intraperitoneally. After recovery, mice were returned to cages and food and water provided. There were 20 mice in each group and were monitored daily over a 10 day period and survival rate recorded.

### Free fatty acid quantification

A colorimetric kit from BioVision, (Milpitas, CA) was used to quantify free fatty acids. Liver tissue was homogenized with chloroform/Triton X-100 and then centrifuged. Chloroform was removed and acyl-CoA was added to the remaining lipid. A reaction mixture was added containing assay buffer, a fatty acid probe, an enzyme and enhancer. Optical density was measured at 570 nm to determine palmitic acid concentration. The assay was performed according to the instructions provided by the manufacturer.

### Determination of serum enzymes and cytokines

To isolate serum, whole-blood samples were centrifuged at 2,000 $g$ for 12 min. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were acquired using Pointe Scientific assay kits (Lincoln Park, MI). Serum and liver tissue interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) levels were quantified by enzyme-linked immunosorbent assay (ELISA) kits from BD Biosciences (San Diego, CA). The assay was executed according to the instructions provided by the manufacturer.

### Assessment of mRNA levels by qPCR analysis

Total RNA was isolated from mouse liver tissues with TRIzol (Invitrogen, Carlsbad, CA). Once extracted, RNA was reverse-transcribed with a murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) into cDNA. PCR was performed using 0.08 μmol each of a forward and reverse primer, cDNA, DEPC water, and 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 μl. Applied Biosystems 7300 real-time PCR machine was used for amplification by a thermal profile of 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Mouse β-actin mRNA was used for normalization. The gene expression data was

shown as fold change from the β-actin level which is calculated as $2^{-\Delta Ct}$. In addition, melting curve analysis was performed to confirm the specificity of PCR product. Relative expression of mRNA levels was expressed as fold change in comparison to the sham tissues. Primers are listed in Table 1.

### RAW 264.7 cell culture and treatment

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC; Manassan, VA). The cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μ/ml streptomycin at 37°C in a humidified atmosphere at 5% $CO_2$. Cells were plated at $1 \times 10^6$ cells in complete medium and treated the next day with various concentrations of C75 in Opti-MEM for 4 h at 37°C in the presence of 10 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO). DMSO (20%) was used as vehicle control for the experiment. The culture supernatant was used for TNF-α measurement.

### Statistical analysis

Data are represented by the mean value ± SE (n=5/group) and were analyzed using one way analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) test for multiple group analyses. The survival rate is estimated by Kaplan-Meier method and compared by the log-rank test. Value differences are considered significant if $P < 0.05$.

## RESULTS

### C75 decreased FFA production in the liver after CLP

The liver is the major the site of FFA production. The FFA levels, as indicated by the measurement of palmitic acid, were significantly increased at 24 h after CLP (Fig. 1). These values were reduced in a dose-dependent manner with C75 treatment. The 1 mg-dose significantly lowered the FFA levels by 28% in comparison with the vehicle. The levels of FFA with 5 mg-dose group were reduced further by 53% from the vehicle (Fig. 1).

### C75 attenuated organ injury indexes after CLP

At 24 h after CLP, serum levels of AST, ALT and LDH were increased by 5.0-, 7.1-, and 7.6-fold in comparison with the sham, respectively (Fig. 2). Treatment with 1 mg-dose after CLP significantly reduced injury marker levels by 50%, 36%, and 65%, while 5 mg-dose reduced these levels by 62%, 66%, and 80% from the vehicle, respectively (Fig. 2).

### C75 reduced serum TNF-α and IL-6 levels after CLP

Overproduction of proinflammatory cytokines can lead to severe organ damage. Serum TNF-α and IL-6 were increased to 144 ± 40 pg/mg protein and 1.5 ± 0.3 pg/mg protein, respectively, after CLP. The 1 mg-dose reduced serum TNF-α and IL-6 levels by 43% and 21%, respectively. Treatment with 5 mg-dose reduced these levels further by 91% and 81%, respectively (Fig. 3).

### C75 reduced liver TNF-α and IL-6 mRNA expression after CLP

In the liver, CLP caused 14- and 43-fold increases in TNF-α and IL-6 mRNA expression which was decreased by 89% and 88%, after treatment with the 1 mg-dose, respectively. Treatment with the 5 mg-dose further reduced these levels to near sham values (Fig. 4).

### C75 decreased liver iNOS and COX-2 mRNA expression after CLP

To determine the effect of C75 on the nitrosative burden caused by CLP, liver tissue levels of iNOS were evaluated 24 h after CLP. Compared with the sham group, we observed a 5-fold increase in iNOS mRNA expression in the vehicle group, which was reduced by 54% and 88% with administration of 1 mg- and 5 mg-dose, respectively (Fig. 5). In addition to iNOS, CLP caused 7.0-fold increase in liver COX-2 mRNA, which was significantly reduced by 46% and 49% with 1 mg- and 5 mg-dose, respectively (Fig. 5).

### C75 improved survival after CLP

The mice treated with 5 mg-dose of C75 at 4 h post-CLP had a better survival rate than the CLP mice treated with vehicle. At day 2, all C75-treated mice survived, but only 90% of the vehicle-treated mice survived. While 45% of the vehicle-treated mice died, only 15% the C75-treated mice died by day 3. The survival rates remained as seen in day 3 for both groups during the 10 day observation period (Fig. 6).

### C75 attenuated LPS-induced TNF-α in macrophages

After observing the reduction of TNF-α level in the CLP mice with C75 treatment, we then examined whether C75 had a direct effect on the TNF-α release in macrophages after stimulation. The murine macrophage RAW 264.7 cells were stimulated with 10 ng/ml LPS for 4 h in the presence of various concentrations of C75. As shown in Fig. 7, the TNF-α levels in the cultured medium of cells treated with 10, 25 and 50 μM of C75 reduced by 24%, 46% and 69%, respectively, in comparison with the vehicle (DMSO) treatment. All C75 doses tested did not affect the viability of the RAW 264.7 cells as determined by MTS assay (data not shown).

## DISCUSSION

The present study showed that administration of C75 at 4 h after CLP significantly decreased liver FFA in a dose-dependent manner, which was correlated with significant decreases in the organ injury markers (AST, ALT, LDH) and serum cytokines (TNF-α, IL-6). The C75 treatment also decreased inflammation and oxidative stress in the liver by decreasing liver cytokines, iNOS and COX-2 gene expression. Finally, treatment with 5 mg-dose of C75 significantly improved survival to 85% from that of 55% in the vehicle. These results clearly indicated that inhibiting lipogenesis in the liver by C75 reduced systemic inflammation, organ injury and mortality after sepsis.

The liver is the major the site of FFA production. FFA are ubiquitous aliphatic acids that play a beneficial role in metabolism, biosynthesis, and cell signaling. However, chronically high levels of FFA can be dangerous. Metabolic abnormalities can arise from surplus fatty acid production which leads to organ failure [19]. Studies have shown that elevated FFA

levels lead to lipotoxicity in a number of organs including the liver [20]. Our studies clearly indicated that C75 treatment significantly decreased sepsis-induced organ injury as evidenced by a significant reduction of liver enzymes (AST, ALT) and LDH. FFA accumulation has been shown to cause inflammatory response through the activation of NF-κB, thereby increasing pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β in the rat liver [21,22]. In the current study, we showed that increase in TNF-α and IL-6 in the liver induced by sepsis were correlated with the increase in FFA levels whereas the decrease of the FFA levels in the liver after C75 treatment is corresponded to the reduction of TNF-α and IL-6 levels. It has also been report that increased FFA levels cause oxidative stress leading to lipid derived free radicals [22,23]. We also showed that increase in FFA levels in the liver could cause elevated iNOS and COX-2 gene expression and that C75 treatment significantly decreased their levels.

Although the mechanism of action of C75 in sepsis-induced liver injury has not been completely elucidated, our data suggest that its action is by inhibiting FFAs and thereby ameliorating lipotoxicity. Recent study also indicated that hepatotoxicity is mostly attributed to FFAs [24]. Critically ill patients present various lipid disorders including high levels of triglycerides, low levels of lipoproteins, and high serum levels of FFAs [25]. Our study has not examined the plasma lipid profile or FFA levels in serum from sepsis animals. Since liver is the major site of FFA production and that we observed significant decrease in FFA in the liver of sepsis animals, it can be speculated that C75 treatment can indeed lower the serum FFA levels.

The targeting of the lipid metabolism can only be beneficial when there is both a modulation of fatty acid synthesis and that of oxidation. C75 inhibits fatty acid elongation thereby increasing the substrate Malonyl CoA. Malonyl CoA is not only a substrate of FAS but also an allosteric inhibitor of CPT-1, the rate limiting enzyme for mitochondrial fatty acid oxidation. Since C75 also stimulates CPT-1, the benefit seen with C75 treatment in sepsis could be attributed to both inhibition of lipogenesis as well as increase in fatty acid oxidation and subsequent energy production in the liver. Further studies are warranted for such notion.

The mechanism by which FFA levels in the liver leads to sepsis-induced injury is yet to be understood. Elevated FFA levels have strongly been associated with inflammation by increasing pro-inflammatory cytokines. In macrophages, FFAs induce cytokines via Toll like 4 receptor and subsequent NF-κB activation [26]. In cultured cardiac cells, palmitate increases p65 translocation and NF-κB activation [27]. Palmitate also increases reactive oxygen species production and oxidative stress in cultured cardiac cells [27]. Inflammation and oxidative stress are strongly associated with apoptosis [28]. Recent study showed that curcumin protected the heart from FFA-induced injury by inhibiting apoptosis via decreasing oxidative stress and inactivating NF-κB [27]. Elevated FFAs also cause necrosis, alter membrane integrity and cell swelling which are indicative of oxidative stress [29,30]. Glycemic control with insulin is a well accepted adjuvant therapy for sepsis patients [31]. While glycemic control lowered the lipoproteins and total cholesterol, FFAs, triglycerides and oxidized low density lipoprotein remained high which suggested that FFA response

continued even under glycemic control [32]. This study suggests that FFA might represent a better therapeutic target in patients with sepsis [32].

The CLP model of sepsis in rodents has been widely used as a model for polymicrobial sepsis [33,34]. In order to establish the $LD_{50}$ model in mice, a single dose of antibiotics, i.e., 0.5 mg/kg BW imipenem is subcutaneously given once immediately after CLP. Without the antibiotics, 100% mortality is observed within 48–72 h after CLP. However, mortality generally is not observed within 24 h time period and therefore antibiotic is routinely not given for such short term studies. C75 is not an antibacterial agent and that it is not known whether C75 treatment reduces bacterial load in the internal organs such as the lungs, liver and blood. If one can assume that the systemic inflammation persists as long as the bacterial load is significant, then our data clearly showed that C75 treatment attenuated systemic inflammation as evidenced by decreases in circulating levels of TNF-α and IL-6 as well as decreases in the gene expression of TNF-α, IL-6, iNOS and COX-2 in the lungs. Therefore, it is possible that C75 treatment decreases the bacterial load in the blood and other internal organs.

One of the concerns in the CLP model is the closing off of the puncture due to local inflammation and thus reducing bacterial insult to the system. However, our data showed that C75 significantly increased injury markers and cytokine levels as compared to sham values. Therefore, it is highly unlikely that the benefit observed with C75 is caused by the reduction in bacterial load due to technical discrepancies of the CLP model. However, additional experiments are needed for such conclusion. It is also not known whether the protective effect seen with C75 treatment is solely due to inhibition of fatty acid synthesis in the liver. However based on our data that C75 treatment decreased the sepsis associated increase in free fatty acids, we speculated that the inhibition of fatty acid synthesis in the liver by C75 could be in part responsible for the observed benefit in the reduction of systemic inflammation and lung injury. However, additional mechanism(s) of its action in sepsis is yet to be elucidated.

All inflammation may not lead to adverse effects and in fact, mild to moderate inflammatory response is rather beneficial. However, one of the phenotypical characteristics of sepsis is the exaggerated inflammatory response and that compounds that can attenuate this effect has shown to be protective in sepsis. We have previously shown protective effects of several such compounds in animal models of sepsis and other organ injury indications [34–36]. It can also be argued that the administration of C75 4 h after CLP can be considered as early treatment and that later time points would have been suitable for testing the therapeutic potential of C75. However, the CLP model employed in this study has already shown significant increases in injury indexes such as AST, ALT and LDH by 24 h suggesting that treatment with C75 at later time point, i.e., 24 h after CLP may or may not be able to reverse the injury associated with sepsis. Therefore, additional experiments addressing C75 treatment at later time points are necessary to determine the therapeutic potential of this compound for sepsis.

## CONCLUSION

Treatment with C75 after CLP reduced FFA levels in the liver and attenuated sepsis associated inflammatory response and mortality. This reduction in FFA could ameliorate the hepatic dysfunction generally seen in sepsis and be in part responsible for the observed benefit of C75 in sepsis.

## ACKNOWLEDGEMENTS

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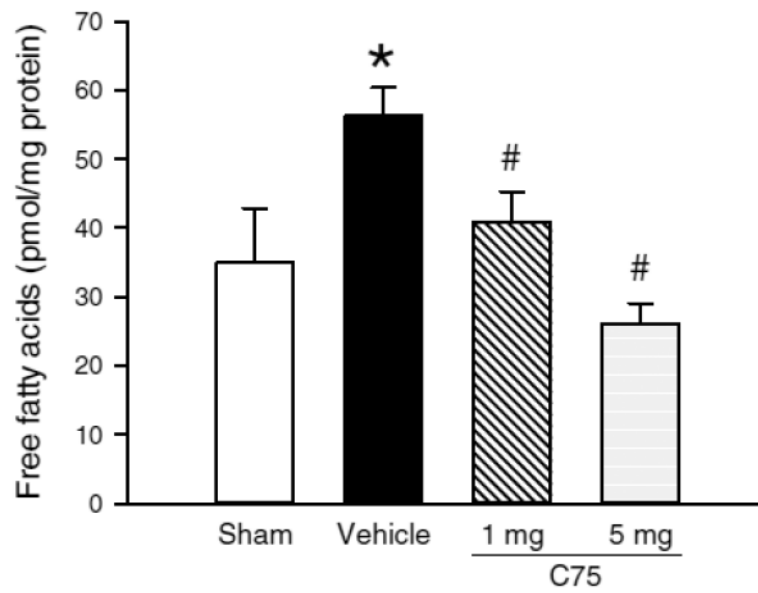
**Figure 1. C75 inhibited liver FFA production after CLP**

Blood and liver tissues were harvested from Sham, Vehicle, C75-treated groups at 24 h after CLP. Liver FFA was measured using a colorimetric assay as described in Methods. Data presented as mean ± SE (n=5/group) and compared by one-way ANOVA and SNK method. *$P < 0.05$ vs. Sham; #$P < 0.05$ vs. Vehicle.
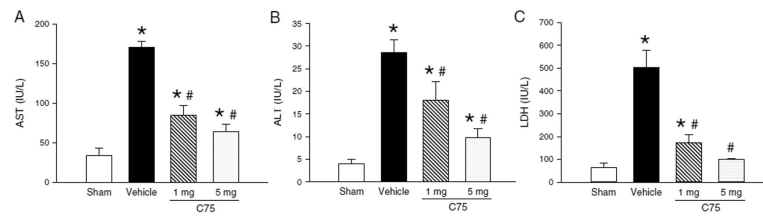
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**Figure 2. C75 reduced serum levels of organ injury indexes after CLP**

Serum samples of Sham, Vehicle, C75-treated groups at 24 h after CLP were measured for (A) AST, (B) ALT, and (C) LDH using commercial kits. Data presented as mean ± SE (n=5/ group) compared by oneway ANOVA and SNK method; *$P$ <0.05 vs. Sham; #$P$ < 0.05 vs. Vehicle.
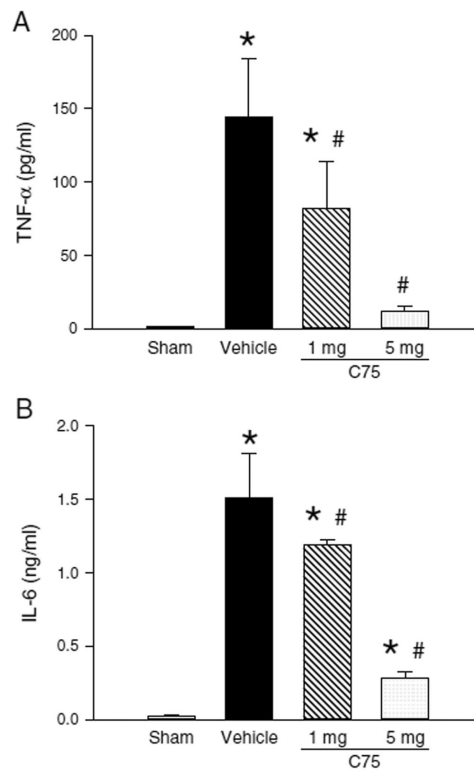
**Figure 3. C75 lowers serum TNF-α and IL-6 proteins after CLP**
Serum samples from Sham, Vehicle, C75-treated groups at 24 h after CLP were determined the levels of (A) TNF-α and (B) IL-6 by ELISA. Data presented as mean ± SE (n=5/group) and compared by one-way ANOVA and SNK method. $*P < 0.05$ vs. Sham and $\#P < 0.05$ vs. Vehicle.
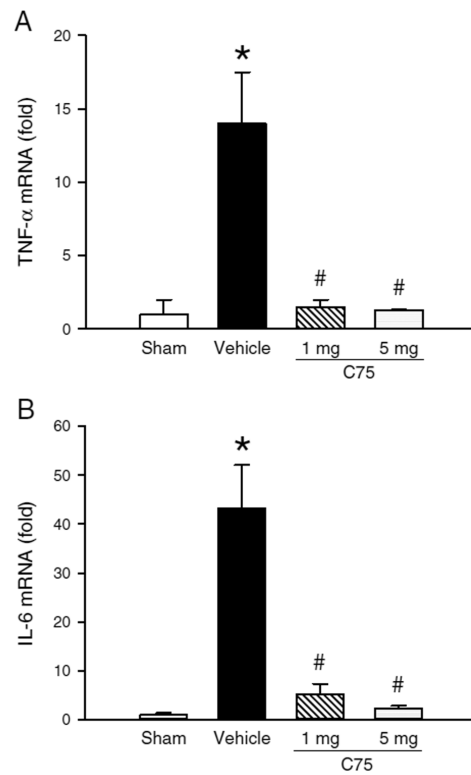
**Figure 4. C75 decreased liver TNF-α and IL-6 mRNA expression after CLP**
Total RNA from liver tissues of Sham, Vehicle, C75-treated groups at 24 h after CLP was extracted and the mRNA levels of (A) TNF-α and (B) IL-6 were determined by real time RT-PCR analysis. Expression levels were normalized with β-actin and the sham value was designated as 1. Data are expressed as mean ± SE (n=5/group) and compared by one-way ANOVA and SNK method. *$P < 0.05$ vs. Sham and #$P < 0.05$ vs. Vehicle.
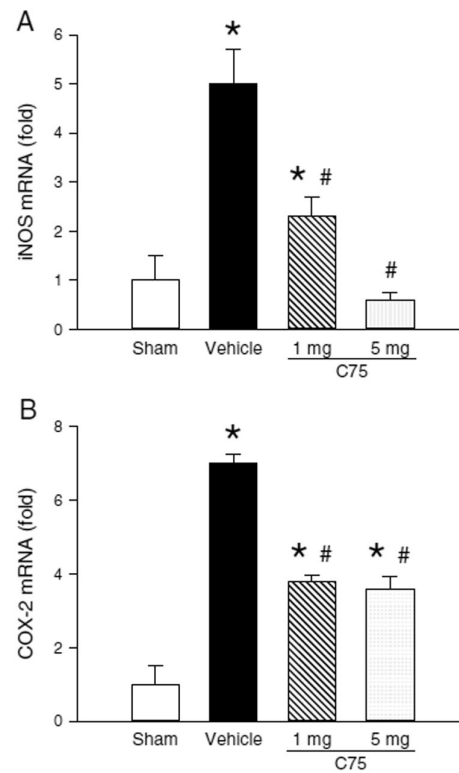
**Figure 5. C75 attenuated liver iNOS and COX-2 mRNA expression after CLP**
Total RNA from liver tissues of Sham, Vehicle, C75-treated groups at 24 h after CLP was
extracted and the mRNA levels of (A) iNOS and (B) COX-2 were determined by real time
RT-PCR analysis. Expression levels were normalized with β-actin and the sham value was
designated as 1. Data are expressed as mean ± SE (n=5/group) and compared by one-way
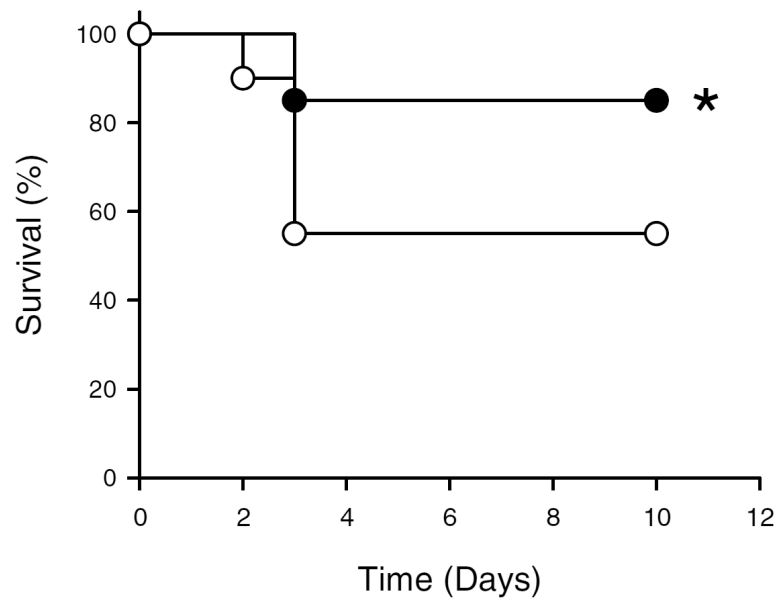ANOVA and SNK method. *$P < 0.05$ vs. Sham.

**Figure 6. C75 treatment improved survival of mice after CLP**
Mice (n=20/group) treated with Vehicle (∘) or 5 mg/kg of C75(•) at 4 h after CLP was monitored daily to assess survival. The survival rate was analyzed by Kaplan-Meier survival analysis and compared by the log-rank test. *$P < 0.05$ vs. Vehicle.
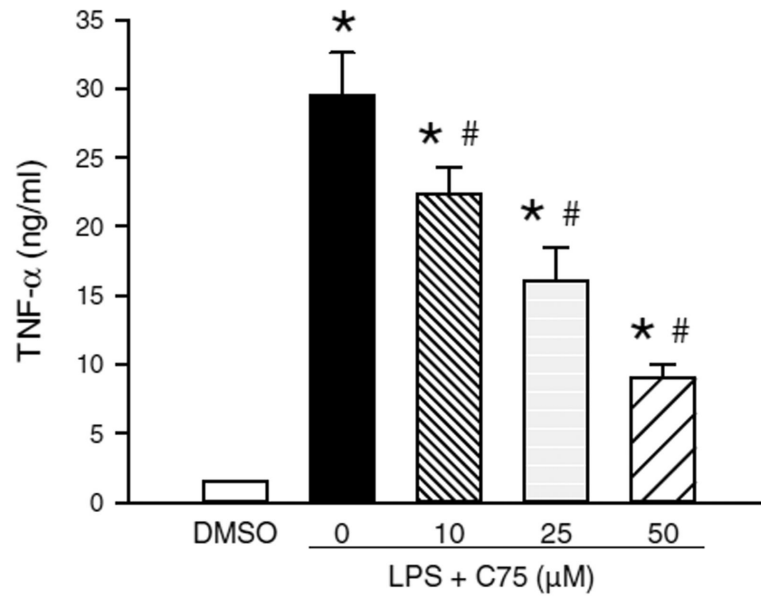
**Figure 7. C75 inhibited TNF-α release in macrophages after LPS stimulation**
RAW 264.7 cells were stimulated with LPS (10 ng/ml) in the presence of the indicated concentration of C75. After 4 h, the cultured medium was collected for the measurement of TNF-α protein levels by ELISA. DMSO was used as the vehicle control. Data are expressed as mean ± SE (n=5/group) and compared by one-way ANOVA and SNK method. *$P < 0.05$ vs. DMSO.

**TABLE 1**

A list of primer sequences used in this study

| Name | GenBank# | Forward | Reverse |
|------|----------|---------|---------|
| TNF-α | X_02611 | AGACCCTCACACTCAGATCATCTTC | TTGCTACGACGTGGGCTACA |
| IL-6 | NM_031168 | CCGGAGAGGAGACTTCACAG | CAGAATTGCCATTGCACAAC |
| COX-2 | NM_011198 | CTCAGCCAGGCAGCAAATC | ACATTCCCCACGGTTTTGAC |
| iNOS | NM_010927 | GCAGGTCGAGGACTATTTCTTTCA | GAGCACGCTGAGTACCTCATTG |
| β-actin | NM_007393 | CGTGAAAAGATGACCCAGATCA | TGGTACGACCAGAGGCATACAG |