Biochemical and Biophysical Research Communications 464 (2015) 774–779

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel natural compound from garlic (*Allium sativum* L.) with therapeutic effects against experimental polymicrobial sepsis

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ARTICLE INFO

Article history:
Received 2 July 2015
Accepted 6 July 2015
Available online 9 July 2015

Keywords:
Sepsis
Sucrose methyl 3-formyl-4-methylpentanoate
Bacteria killing
Inflammation

ABSTRACT

Sepsis is a serious, life-threatening, infectious disease. In this study, we demonstrate that sucrose methyl 3-formyl-4-methylpentanoate (SMFM), a novel natural compound isolated from garlic (Allium sativum L.), markedly enhances survival rates by inhibiting lung inflammation in a cecal ligation and puncture (CLP) experimental polymicrobial sepsis model. SMFM strongly reduced bacterial colony units from peritoneal fluid in CLP mice by stimulating the generation of reactive oxygen species. Lymphocyte apoptosis in spleens from CLP mice was also markedly decreased by SMFM administration. SMFM also significantly inhibited the production of proinflammatory cytokines, such as TNF-α, interleukin-1β (IL-1β) and IL-6, in CLP mice. Lipopolysaccharide-stimulated production of TNF-α and IL-6 were also strongly inhibited by SMFM in mouse bone marrow-derived macrophages. Taken together, our results indicate that SMFM has therapeutic effects against polymicrobial sepsis that are mediated by enhanced microbial killing and blockage of cytokine storm.

1. Introduction

Sepsis is a serious, life-threatening infectious disease [1]. Causes of sepsis outbreaks include infection with Gram negative bacteria, Gram positive bacteria, fungi and so on [1]. During the pathological process of sepsis, a hyperimmune response is induced during the early stage by pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), resulting in the production of too many proinflammatory mediators, such as tumor necrosis factor (TNF) and interleukin (IL)-1β. Later in the course of the disease, these accumulated proinflammatory mediators cause lymphocyte apoptosis and organ damage, resulting in immune paralysis and increased mortality [1,2]. According to previous reports, approximately 750,000 patients develop sepsis annually in the United States [3,4]. The incidence rate of sepsis is gradually increasing; from 300,000 to approximately 800,000 from 2000 to 2007 [3,4]. Although the mortality of sepsis is still too high at approximately 27% of septic patients [3,4], no effective therapeutic drugs are clinically available. Thus, the development of therapeutic molecules to treat sepsis has been the focus of much research.

Although garlic (*Allium sativum* L.) has been used as a food, spice, and herbal medicine for several decades in many countries [5,6], extensive scientific investigation to purify bioactive molecules from garlic and characterize them has been conducted only relatively recently [7–10]. According to previous studies, garlic has helpful effects on the treatment of diverse human diseases, including infections, some kinds of cancer, and cardiovascular disorders [7–10]. Because the putative therapeutic effects of garlic extracts or garlic-derived molecules against sepsis have not been fully investigated, in this study we isolated a novel natural compound that inhibits LPS-induced inflammatory cytokine production, revealed structural information of the compound, and examined the in vivo efficacy of the garlic-derived molecule in an experimental polymicrobial sepsis model.

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http://dx.doi.org/10.1016/j.bbrc.2015.07.031
0006-291X/

2. Materials and methods

2.1. Materials

Garlic cloves were purchased directly from New Green Company (Changnyeong, Korea) in January, 2008. The garlic cloves used in this study were cultivated in Changnyeong, Korea. A voucher specimen was deposited at the Plant Drug Research Laboratory, Pusan National University, Korea (accession number GA-PRDR-11). Hexane, chloroform, ethyl acetate, and methanol were purchased from Fisher Scientific, Ltd. (Pittsburgh, PA, USA).

2.2. General experimental procedures

Optical rotations were recorded on a JASCO DIP-370 digital polarimeter. NMR spectra (^{1}H, ^{13}C, COSY, HMQC, and HMBC) were recorded in CD_3OD on an NMR (Varian Inova 500, Santa Clara, CA, USA) spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, running gradients and using residual solvent peaks as internal references. High-resolution mass spectra were recorded on a Q-TOF (Agilent, Q-TOF 6530/AB Sciex, Q-TOF 4600, Santa Clara, CA, USA) mass spectrometer.

2.3. Purification of sucrose methyl 3-formyl-4-methylpentanoate (SMFM)

Freeze-dried cloves (NGLF, 1039.63 g) were ground to a fine powder and successively extracted with n-hexane, ethyl acetate, and methanol at room temperature. The methanol extract (NGLFM, 137.07 g) of 50.45 g was evaporated under vacuum and chromatographed on silica gel (40 μm; Baker; Phillipsburg, NJ, USA) columns (80 cm × 5.5 cm) with a step gradient, running the following solvents: 50% acetone in chloroform, 50, 60, and 75% methanol in chloroform and 45% methanol, 9% dH_2O and 0.25% acetic acid in chloroform to get NGLFM34 (1.216 g). The NGLFM34 fraction was evaporated in vacuo and dissolved with chloroform. The dissolved NGLFM34 fraction was stand at room temperature for 1 day, and washed precipitated compound with chloroform to get pure NGLFM34 (i.e. SMFM) (78 mg).

2.4. Cytokine release from bone marrow-derived macrophages

Mouse bone marrow-derived macrophages were generated as described previously [11]. Mouse bone marrow-derived macrophages were incubated with vehicle (0.1% DMSO in PBS) or SMFM (200 μg/ml) for 30 min. Then the cells were stimulated with PBS or LPS (100 ng/ml) for 4 or 12 h. After collecting cell-free supernatants, the levels of TNF-α and IL-6 were measured by ELISA (BD Biosciences Pharmingen).

2.5. Measurement of IκB degradation by Western blot

Mouse bone marrow-derived macrophages were incubated with vehicle (0.1% DMSO in PBS) or SMFM (200 μg/ml) for 15 min. Then the cells were stimulated with LPS (100 ng/ml) for 30 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The levels of IκB and actin were detected using antibodies against each protein.

2.6. Animals and sepsis model

To test whether SMFM had any therapeutic effect against sepsis, we conducted experiments using a cecal ligation and puncture (CLP) model using six week old male wild type ICR mice, as described previously [12,13]. After ligating the cecum below the ileocecal valve, we punctured through both surfaces twice using a 22-gauge needle, and the abdomen was closed. For the measurement of cytokine production or bactericidal activity, we punctured once. Survival was monitored once daily for 10 days.

2.7. Tissue histology

ICR mice were subjected to CLP surgery. Vehicle (0.8% DMSO in PBS) or SMFM (15 mg/kg) was administered 2 and 14 h after CLP. The mice were killed 24 h after surgery, after which their lungs were fixed, sectioned, and stained with hematoxylin and eosin for morphological analysis.

2.8. Measurement of bactericidal activity in CLP model

Twenty-four hours after CLP, we collected peritoneal lavage fluid and cultured it overnight on blood-agar base plates (Trypticase Soy Agar Deeps; Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C. We then counted colony forming units (CFUs) from the samples, as described previously [13].

2.9. Measurement of superoxide anion production from neutrophils

Mouse neutrophils were isolated using a previously reported method [14]. Freshly isolated mouse neutrophils (2×10^6 cells in RPMI 1640 medium) were preincubated with 50 μM cytochrome c at 37 °C for 5 min and subsequently incubated with vehicle (0.1% DMSO in PBS) or SMFM (200 μg/ml). Superoxide generation was determined by measuring light absorption changes at 550 nm over 8 min, at 1 min intervals, as described previously [15].

2.10. Measurement of apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Twenty-four hours after CLP, spleens were isolated from mice. The TUNEL assay was performed using frozen tissue sections prepared according to a standard histological protocol. The percentage of apoptotic (TUNEL-positive) cells was measured by counting 500 cells under a light microscope, as described previously [13].

2.11. Cytokine measurement

Peritoneal lavage fluids were collected from mice 24 h after CLP. The levels of cytokines present in the peritoneal fluid were determined using an enzyme-linked immunosorbent assay (ELISA; BD Biosciences Pharmingen, San Jose, CA, USA), as described previously [13].

2.12. Statistical analyses

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

3. Results

3.1. Identification of a novel compound that inhibits LPS-stimulated proinflammatory cytokine production in macrophages

In this study, we tested several garlic extracts to identify molecules that inhibit LPS-induced proinflammatory cytokine production in bone marrow-derived macrophages. Among several garlic extracts, methanol extracts contain bioactive molecules that inhibit TNF-α production induced by LPS. By performing sequential

experiments, we successfully isolated a pure compound that inhibits LPS-induced TNF-α production in macrophages.

The novel compound isolated from garlic cloves was obtained as a white sticky with $[\alpha]^{25.4}_{D}$ −69.56 (*c* 0.23, MeOH). The positive Tof-MS indicated a molecular ion at m/z 493.3361 corresponding to $[M+2Na-H_2O]^+$, thus indicating a molecular formula of $C_{19}H_{32}O_{13}$. Analysis of 1D- and 2D-NMR data with homo- and heteronuclear direct and long-range correlations permitted assignments of the ^1H and ^{13}C NMR resonances as listed in Table 1.

From the molecular ion signal at m/z 468 of compound, the elemental composition of $C_{19}H_{32}O_{13}$ was determined. This finding was confirmed by the ^1H, ^{13}C, and Dept NMR technique, which showed two primary (δ_C 16.80, 18.13), four secondary (δ_C 30.42, 60.29, 61.53, 62.53), ten tertiary (δ_C 29.25, 60.57, 69.40, 71.24, 72.58, 72.74, 74.17, 76.59, 81.54, 92.35), and three quaternary carbons (δ_C 103.85, 174.41, 215.61). The ^1H NMR spectrum of the novel compound exhibited signals for two-methyl (δ_H 0.82, d, J = 7.0, H-5′/0.87, d, J = 7.0, H-6′), four-methylene (δ_H 2.07, t, H-2′/3.51, t, H-10, H-12/3.65, t, H-11, H-12), and ten-methine (δ_H 2.11, m, H-4′/3.30, t, H-2/3.39, dd, *J* = 10.0/4.0, H-1/3.44, d, *J* = 4.5, H-3′/3.59, t, H-3/3.65, t, H-4/3.72, m, H-9/3.88, t, H-8/4.05, d, *J* = 8.5, H-7/5.25, d, *J* = 4.0, H-5) group protons. Their connectivities were established from the long-range $^1H-^{13}C$ correlations observed in the HMBC spectrum (Fig. 1A, B and Table 1).

The interpretation of all these spectra of compound has revealed the presence of three structural elements; $-CH_2$-CH−CH−CH−CH−CH (glucose moiety), −CH−CH−CH−CH_2- (fructose moiety), and $(CH_3)_2$−CH−CH(COH)−CH_2−C═O−O−CH_2 (methyl 3-formyl-4-methylpentanoate moiety). The positions of the methyl 3-formyl-4-methylpentanoate ester groups could also be unambiguously determined by the HMBC experiment and COSY spectral data. The corresponding cross peaks appear in the $^1H^1H$-COSY (data not shown). The connectivity of the different groups was established through two-dimensional NMR experiments. The $^1H^1H$-COSY revealed the presence of an isopropyl group due to identical cross peaks to the multiplet of H-4′/H-5′. This finding was further confirmed by the HMBC in which carbon H-3′ couples to both methyl doublets C-1′, C-4′, and C-6′. The same applies to carbon H-2′, which also couples to aldehyde (C-7′) and secondary carbon (C-3′). As H-5′ and H-6′ couples to the C-3′ and 4′, the connectivities in the methyl 3-formyl-4-methylpentanoate established. From this information, the connectivity of a SMFM was deduced. The absolute configuration of the novel compound was established, as shown in Fig. 1A and B.

Table 1
^1H (500 MHz in CD_3OD) and ^{13}C NMR (150 MHz in CD_3OD) and HMBC data of sucrose methyl 3-formyl-4-methylpentanoate (SMFM).

Position	δ_C		δ_H	HMBC
1	71.24	CH	3.39, dd, 10.0/4.0	
2	69.40	CH	3.30, t	72.74, 60.29
3	72.74	CH	3.59, t	
4	72.58	CH	3.65, t	
5	92.35	CH	5.25, d, J = 4.0	103.85, 72.58
6	103.85	C		
7	76.59	CH	4.05, d, J = 8.5	74.17
8	74.17	CH	3.88, t	62.53
9	81.54	CH	3.72, m	74.17
10	61.53	CH_2	3.51, s	103.85, 76.59
11	60.29	CH_2	3.65, t	
12	62.53	CH_2	3.65, t	81.54, 69.40
			3.51, t	81.54, 69.40
1′	174.41	C		
2′	30.42	CH_2	2.07, t	215.64, 60.57
3′	60.57	CH	3.44, d, J = 4.5	174.41, 29.25, 16.18, 18.13
4′	29.25	CH	2.11, m	18.13, 16.80
5′	18.13	CH_3	0.87, d, J = 7.0	60.57, 29.25, 16.80
6′	16.80	CH_3	0.82, d, J = 7.0	60.57, 29.25, 18.13
7′	215.61	C		

Stimulation of mouse bone marrow-derived macrophages with 100 ng/ml LPS strongly increased the production of proinflammatory cytokines, such as TNF-α and IL-6 (Fig. 1C, D). SMFM (200 μg/ml) strongly decreased the levels of proinflammatory cytokines induced by LPS in these mouse bone marrow-derived macrophages (Fig. 1C, D). With respect to the production of proinflammatory cytokines, such as TNF-α and IL-6, NF-κB transcription factor activity is essential [16]. Since SMFM strongly blocked LPS-induced proinflammatory cytokine production, we tested the effect of SMFM on LPS-stimulated NF-κB activation. NF-κB activation is accompanied by IκB degradation [17]. We also found that stimulation of mouse bone marrow-derived macrophages with LPS elicited IκB degradation (Fig. 1E). However, SMFM partially inhibited LPS-stimulated IκB degradation (Fig. 1E). These results suggest that SMFM may inhibit LPS-stimulated NF-κB activation, and the subsequent production of proinflammatory cytokines.

3.2. Administration of SMFM increases survival rate in CLP sepsis by enhancing bacterial clearance

In this study, we examined whether SMFM had a therapeutic effect against experimental polymicrobial sepsis using the CLP model. Use of a severe CLP model caused a dramatic decrease in the survival rate of mice and caused 100% mortality at day 2 (Fig. 2A). However, administration of SMFM at two different dosages (10 mg/kg and 15 mg/kg) enhanced survival rate. When 15 mg/kg SMFM was administrated 2 h post CLP and at 12 h intervals an additional three times, the survival rate increased, reaching 54.5% (Fig. 2A). Lung inflammation is known to be closely associated with the pathogenesis of sepsis [1,2]. We also found that CLP induced lung inflammation, resulting in severe alveolar congestion and extensive thrombotic lesions in the lung (Fig. 2B). CLP-induced lung inflammation was markedly inhibited by the administration of SMFM (Fig. 2B).

Sepsis is caused by infection by several different pathogens, including bacteria [1]. Because CLP surgery causes the release of very diverse intestinal contents, including viable bacteria, the experimental CLP sepsis model mimics human infection-induced sepsis [18]. Lethality caused by CLP surgery is associated with increased bacterial colony counts in peritoneal fluid [18]. Here we examined the effect of SMFM administration on bacterial colony counts in CLP sepsis. CLP surgery markedly increased bacterial colony counts in peritoneal fluid; these counts decreased dramatically upon administration of SMFM (Fig. 2C). This result correlates well with SMFM-induced anti-septic activity in terms of increased survival rate (Fig. 2A). Since bactericidal activity in the CLP sepsis model is mediated by the production of reactive oxygen species, such as superoxide anion in phagocytic cells, we investigated the effect of SMFM on the production of superoxide anion in mouse neutrophils. As expected, stimulation of mouse neutrophils strongly increased production of superoxide anions, reaching maximal activity at 200 μg/ml (Fig. 2D). This result suggests that SMFM strongly stimulates bacterial killing activity by enhancing the production of reactive oxygen species in phagocytic cells.

3.3. SMFM administration markedly inhibits CLP-induced lymphocyte apoptosis and proinflammatory cytokines

Because lymphocyte apoptosis in the spleen are markedly induced during the pathogenesis of sepsis, leading to immune paralysis in sepsis patients, we investigated whether SMFM had any effect on this process. CLP surgery caused increases in apoptosis, as

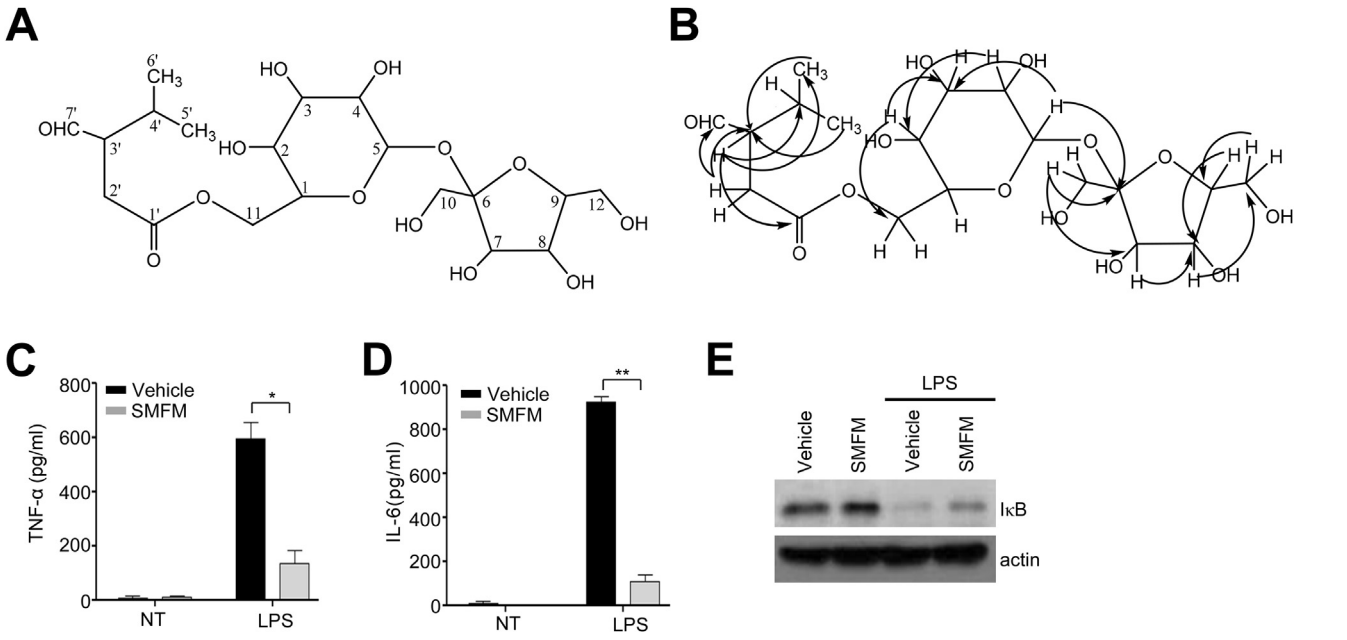

Fig. 1. Effects of SMFM on LPS-induced cytokine production. (A) The chemical structure of SMFM. (B) Key HMBC correlations of SMFM. (C, D) Mouse bone marrow-derived macrophages were preincubated with vehicle (0.1% DMSO in PBS) or SMFM (200 μg/ml) for 30 min, and subsequently stimulated with LPS (100 ng/ml) for 4 (for IL-6) or 12 h (for TNF-α). TNF-α (A), and IL-6 (B) levels were measured by ELISA. Data are expressed as the mean ± SEM (n = 8). *, $P < 0.05$; **, $P < 0.01$ (C,D). (E) Mouse bone marrow-derived macrophages were preincubated with vehicle (0.1% DMSO in PBS) or SMFM (200 μg/ml) for 15 min, and then stimulated with LPS (100 ng/ml) for 30 min. The levels of IκB and actin were measured by Western blot analysis. Data are representative of two independent experiments (E).

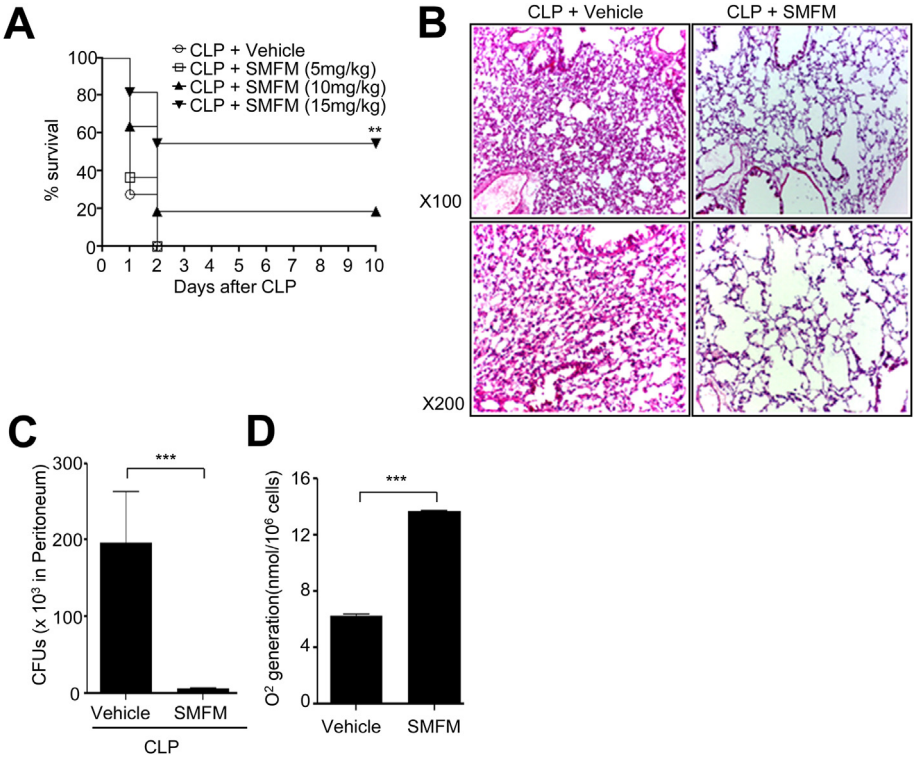

Fig. 2. SMFM protects against CLP-induced mortality by stimulating bactericidal activity. Several different doses (0, 5, 10, 15 mg/kg) of SMFM were subcutaneously injected into CLP mice at 2, 14, 26, and 38 h post-CLP (A). Vehicle (0.8% DMSO in PBS) or SMFM (15 mg/kg) was administered 2 and 14 h after CLP. The mice were sacrificed 24 h after surgery and their lungs were stained with hematoxylin and eosin (magnification, × 100 or × 200) (B). **$P < 0.005$ compared to vehicle control by ANOVA (A). Sample size: n = 11 mice/group (A). The data are representative of eight mice per group (B). (C) Vehicle (0.8% DMSO in PBS) or SMFM (15 mg/kg) was injected into CLP mice 2 and 14 h post-CLP. Peritoneal lavage fluid collected 24 h after CLP or CLP plus SMFM administration was cultured overnight on blood-agar base plates at 37 °C, and the resulting CFUs were determined. (D) Freshly isolated mouse neutrophils were stimulated with vehicle (0.1% DMSO in PBS) or SMFM (200 μg/ml) for 8 min. The amount of superoxide anion produced from neutrophils was measured using a cytochrome c reduction assay. Data are expressed as the mean ± SEM; n = 8 (C, D). ***, $P < 0.001$.

detected by DNA fragmentation analysis (TUNEL assay), of splenocytes, (Fig. 3A). However, administration of SMFM dramatically inhibited apoptosis of splenocytes in CLP mice (Fig. 3A). The number of apoptotic cells decreased by 48.4% upon administration of SMFM (Fig. 3B).

During the pathogenesis of sepsis, too many proinflammatory cytokines are produced and this cytokine storm elicits vital organ damage [1,2]. In this study we also observed that CLP surgery markedly increased the production of proinflammatory cytokines important in CLP pathogenesis, such as TNF-α, IL-6, and IL-1β (Fig. 3C–E). We tested the effect of SMFM on the production of proinflammatory cytokines using peritoneal fluid in CLP mice. Administration of SMFM 2 h post-CLP and again at 14 h post-CLP, strongly inhibited the production of important proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β (Fig. 3C–E).

4. Discussion

Although the incidence of sepsis has been gradually increasing and mortality is too great, no effective therapeutic agents are available. Given these circumstances, it has been a hot topic of research to identify molecules that can be used to treat sepsis. In this study we demonstrated that SMFM, a novel natural compound isolated from garlic (Allium sativum L.), showed a strong therapeutic effect against experimental sepsis. SMFM-induced antiseptic activity was accompanied by increased bactericidal activity, inhibition of leukocyte apoptosis in the spleen, and inhibition of proinflammatory cytokines in vivo and in vitro. We think that in vitro dosage used in this study might be pharmacologically relevant to the dosages used for in vivo experiments.

Sepsis-induced mortality is closely associated with vital organ damage [1,2]. We also observed that experimental sepsis induced lung inflammation and damage, causing marked alveolar congestion and extensive thrombotic lesion formation in the lung. Previous reports showed that bacteria released from cecum enter the blood stream, thereby also distributing bacteria into the lung [19,20]. In this study we demonstrated that SMFM elicited strong bactericidal activity in CLP experimental sepsis, and induced superoxide anion production in mouse neutrophils (Fig. 2). Because superoxide anion is an important weapon in killing bacteria [21], SMFM-stimulated bactericidal activity may be mediated by superoxide anion production. Our results suggest that the inhibitory effect of SMFM on lung inflammation and damage can be mediated by the enhanced bactericidal activity of SMFM. We also found that SMFM strongly blocked the production of several important proinflammatory cytokines, including TNF-α, IL-6, IL-1β (Figs. 1 and 3). These proinflammatory cytokines contribute to lung inflammation and damage [1,2]. Therefore, the inhibitory activity resulting from the production of these proinflammatory cytokines induced by CLP or LPS may contribute to the protective effect of SMFM against CLP-induced lung damage.

LPS, an important pathogen-associated molecular pattern of Gram negative bacteria, binds to TLR4 on inflammatory cells, and induces a complex signaling pathway which results in the

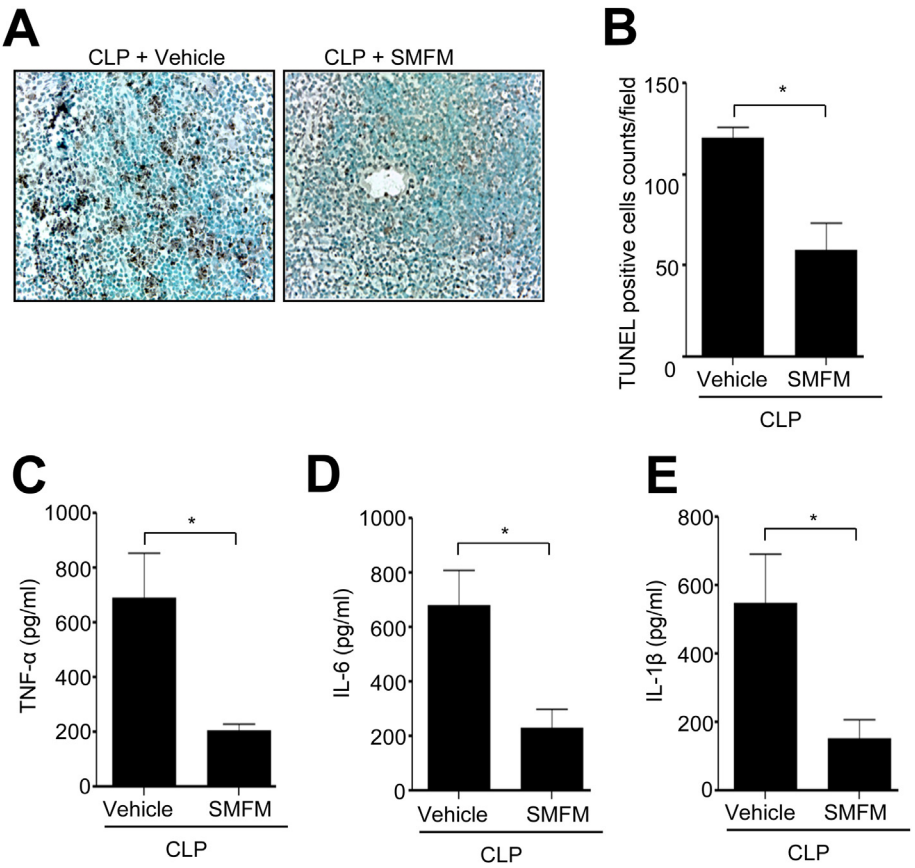

Fig. 3. SMFM inhibits CLP-induced leukocyte apoptosis and proinflammatory cytokine production. (A) Vehicle (0.8% DMSO in PBS) or SMFM (15 mg/kg) was injected into CLP mice 2 and 14 h post-CLP. The spleens were collected 24 h after sham, CLP plus vehicle, or CLP plus SMFM administration, and used in a TUNEL assay. Data are representative of eight mice per group (A). (B) TUNEL-positive cells from the spleens of the mice described in (A) were quantified. (C–E) Vehicle (0.8% DMSO in PBS) or SMFM (15 mg/kg) was injected twice into CLP mice at 2 and 14 h post-CLP. Separate groups of animals were subjected to CLP plus vehicle or CLP plus SMFM treatment. Peritoneal fluids were collected 24 h after CLP. Cytokine levels for TNF-α, IL-6, and IL-1β in the peritoneal fluid were determined by ELISA analysis. Data are expressed as the mean ± SEM (n = 8). *, $P < 0.05$ (B–E).

regulation of several important cytokines [22,23]. NF-κB plays a key role downstream of LPS signaling in the regulation of gene expression of proinflammatory cytokines [22,23]. As we found that LPS-stimulated proinflammatory cytokine production was strongly blocked by SMFM in macrophages, we assume that SMFM may block a signaling pathway induced by LPS in cells. We found that stimulation of macrophages with SMFM blocked LPS-induced TLR4-mediated NF-κB activity and, more precisely, IκB degradation (Fig. 1E). These results suggest that SMFM may be an important molecule that negatively regulates LPS-induced signaling and inflammatory responses.

In conclusion, a novel natural compound isolated from the garlic (Allium sativum L.), SMFM, had therapeutic effects against experimental sepsis by stimulating bactericidal activity, inhibiting lymphocyte apoptosis, and regulating cytokine expression. SMFM and its unidentified target(s) may be usefully in the development of effective anti-septic agents and anti-inflammatory agents.

Acknowledgments

This paper was supported by Samsung Research Fund, Sungkyunkwan University, 2013.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.031.

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