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Suppressive effects of rare ginsenosides, Rk1 and Rg5, on HMGB1-mediated septic responses

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ABSTRACT

High mobility group box 1 (HMGB1) is considered to be a late mediator of sepsis. The inhibition of HMGB1-mediated severe inflammatory response and restoration of endothelial integrity have emerged as attractive therapeutic strategies for the management of sepsis. Rare ginsenosides, Rk1 (**SB1**) and Rg5 (**SB2**), are among the main components of black ginseng and are prepared from ginsenoside Rd by steaming at 120 °C for 3 h. We examined the effects of **SB1** and **SB2** on HMGB1-mediated septic response and survival rate in a mouse model of sepsis. **SB1** and **SB2** were administered after challenge with HMGB1. **SB1** and **SB2** significantly reduced the release of HMGB1 in lipopolysaccharide (LPS)-activated primary human umbilical vein endothelial cells (HUVECS) via the SIRT1-mediated deacetylation of HMGB1. Moreover, **SB1** and **SB2** suppressed the production of TNF-α and IL-6 and the activation of NF-κB and ERK 1/2 by HMGB1. **SB1** and **SB2** also inhibited HMGB1-mediated hyperpermeability and leukocyte migration in mice. In addition, treatment with **SB1** and **SB2** reduced the cecal ligation and puncture-induced release of HMGB1, sepsis-related mortality, and tissue injury in vivo. Our results indicate that **SB1** and **SB2** might be useful in the treatment of sepsis by targeting HMGB1.

1. Introduction

Sepsis is defined as a systemic inflammatory response syndrome, which is caused by infection and remains a common cause of morbidity and mortality, despite recent advances in antibiotic therapy and intensive care (Russell, 2006). It has previously been demonstrated that a persistent increase in plasma levels of the cytokine high mobility group box 1 (HMGB1) in septic patients is correlated with the degree of organ dysfunction and eventually patient outcome (Gibot et al., 2007; Sunden-Cullberg et al., 2005; Wang et al., 2001). In contrast to early inflammatory mediators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, HMGB1 is a late mediator of sepsis and correlates with patient prognosis (Abraham et al., 2000). In response to infection or injury, HMGB1 is actively secreted by innate immune cells and/or is passively released by injured or damaged cells (Bae, 2012; Ulloa and Tracey, 2005). The secreted HMGB1 can trigger a lethal inflammatory process by significantly increasing the release of inflammatory cytokines, as well as by enhancing the expression of cell adhesion molecules, which promote inflammation via the recruitment of leukocytes (Andersson et al., 2000; Bae and Rezaie, 2011). As a late inflammatory mediator of sepsis, HMGB-1 provides a wide therapeutic window for clinical intervention and, therefore, remains an attractive target for sepsis treatment (Bae, 2012).

Ginseng, the radix of *Panax ginseng* Meyer, known as Korean ginseng, is one of the most ancient herbs in traditional medicine and is widely used. Ginseng has been documented to possess various biological activities such as anti-fatigue, anti-cancer, anti-viral, and stress-resistance properties (Kang et al., 2017; Lee et al., 2015; Oh et al., 2015; Wu et al., 2017). Ginsenosides, the main ingredients of ginseng, are classified based on their steroidal structure and number of hydroxyl

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groups/sugar moieties attached to it; these include protopanaxadiol and protopanaxatriol. Among them, ginsenosides, Rk1 (**SB1**) and Rg5 (**SB2**), are the major protopanaxadiol-type components of black ginseng (Cho et al., 2016). To the best of our knowledge, few studies are available on the in vivo protective effect of **SB1** and **SB2** against HMGB1-induced septic response. Herein, we report the in vivo and in vitro antiseptic effects of **SB1** or **SB2** on HMGB1-mediated septic responses.

2. Materials and methods

2.1. Reagents

Bacterial lipopolysaccharide (LPS, serotype: 0111:B4, L5293), sulforaphane (SFN), Evans blue, crystal violet, 2-mercaptoethanol, and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan) and fetal bovine serum and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA).

2.2. Preparation of *SB1* and *SB2*

The rare ginsenosides, Rk1 (**SB1**) and Rg5 (**SB2**), which are among the main components of black ginseng were prepared by the previously reported method from ginsenoside Rd by steaming at 120 °C for 3 h (Sun et al., 2009a).

2.3. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and were maintained in culture as described previously (Jung et al., 2016). These cells were used at culture passages 3–5. Human neutrophils were freshly isolated from whole blood (15 mL) obtained from five healthy volunteers by venipuncture and were maintained as previously described (Jung et al., 2016).

2.4. Animals and cecal ligation and puncture (CLP)

Male C57BL/6 mice (6–7 weeks old, 27 g) purchased from Orient Bio Co. (Sungnam, Republic of Korea) were maintained as previously described (Bae et al., 2014). To induce sepsis, male mice were anesthetized with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg/kg) and Rompum (xylazine, 10 mg/kg). The CLP-induced sepsis model was prepared as previously described (Bae et al., 2014; Wang et al., 2004). All the animals were treated in accordance with the "Guidelines for the Care and Use of Laboratory Animals" issued by Kyungpook National University (IRB No. KNU 2016-54).

2.5. Competitive enzyme-linked immunosorbent assay (ELISA) for HMGB1

A competitive ELISA was performed to determine the HMGB1 concentrations in the cell culture medium or in mice serum, as previously described (Jung et al., 2016). The HUVEC monolayers were treated first with LPS (100 ng/mL) for 16 h and then with each compound for 6 h. After treatment, the cell culture medium was collected for the determination of HMGB1.

2.6. Preparation of cytoplasmic and nuclear extracts

The cells were harvested rapidly by sedimentation and nuclear and cytoplasmic extracts were prepared on ice, as previously described (Mackman et al., 1991). Briefly, the cells were harvested and were washed with 1 mL of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 19 mM KCl) for 5 min at 600 × *g*. Subsequently, the cells were resuspended in buffer A, centrifuged at 600 × *g* for 3 min, resuspended in 30 μL of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA), rotated for 30 min at 4 °C, and centrifuged at 13,000 × *g* for 20 min. The supernatant was used as the nuclear extract. The nuclear and cytosolic extracts were analyzed for protein content using the Bradford assay.

2.7. Immunoprecipitation and western blotting

Cells were lysed in 200 μL of Pierce immunoprecipitation (IP) Lysis Buffer (Thermo Fisher Scientific) and centrifuged for 15 min at 15,000 × *g* at 4 °C. After extraction, 200–300 μg of total cellular protein was precleared with Protein G Sepharose 4 Fast Flow (GE Healthcare Life Sciences, Buckinghamshire, UK) for 1 h at 4 °C and then briefly centrifuged. The precleared cellular lysate was incubated with anti-HMGB1 (Santa Cruz Biotechnology) overnight at 4 °C with constant rotation and then incubated for 4 h with Protein G agarose. After centrifugation, the Sepharose beads were washed with PBS and prepared for western blot analysis.

For western blotting of NF-κB and ERK 1/2, anti-phospho-ERK1/2, anti-total ERK1/2, anti−NF−κB p65, and anti-lamin B or β-actin antibodies (Santa Cruz) were used. β-actin or lamin B was used as a loading control for cytoplasmic and nuclear extracts, respectively.

2.8. Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was used as an indicator of cell viability as previously described (Jung et al., 2016; Kim and Bae, 2016). The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium and incubated for 48 h with each compound.

2.9. In vitro permeability assay

For the spectrophotometric quantification of endothelial cell permeability in response to increasing concentrations of each compound, the flux of Evans blue-bound albumin across functional cell monolayers was measured using a modified two-compartment chamber model, as previously described (Bae and Rezaie, 2011). The HUVECs were plated (5×10^4 cells/well) in transwells (pore size, 3 μm; diameter, 12 mm) for 3 days. Confluent monolayers of HUVECs were first treated with LPS (100 ng/mL) for 4 h or with HMGB1 (1 μg/mL) for 16 h, which was followed by subsequent treatment with each compound.

2.10. Cell–cell adhesion assay

Purified human neutrophils (1.5×10^6 cells/mL, 200 μL/well) were labeled with Vybrant DiD dye and then added to washed and stimulated HUVECs. The HUVEC monolayers were first treated with HMGB1 (1 μg/mL) for 16 h and then with each compound for 6 h. The neutrophils were allowed to adhere and the non-adherent neutrophils were removed by washing. The percentage of adherent neutrophils was calculated using the formula: % adherence = (adherent signal/total signal) × 100.

2.11. In vitro migration assay

Migration assays were performed in transwell plates (diameter, 6.5 mm) that contained filters with a pore size of 8 μm. The HUVECs (6×10^4) were cultured for 3 days to obtain confluent endothelial monolayers. Prior to the addition of neutrophils to the upper compartment, cell monolayers were treated with HMGB1 (1 μg/mL) for 16 h, followed by treatment with each compound for 6 h. The transwell plates were then incubated at 37 °C in 5% CO_2 for 2 h. The cells in the upper chamber were aspirated and the non-migrating cells on the top of the filters were removed using a cotton swab. The neutrophils on the lower side of the filter were fixed in 8% glutaraldehyde and stained

with 0.25% crystal violet in 20% methanol (w/v). The experiments were repeated twice per well in duplicate wells, and nine randomly selected high power microscopic fields (HPF, 200 ×) were counted. The results are presented as migration indices.

2.12. In vivo permeability and the leukocyte migration assay

For the in vivo study, male mice were anesthetized with 2% isoflurane (Forane, JW Pharmaceutical, South Korea) in oxygen delivered via a small rodent gas anesthesia machine (RC2, Vetequip, Pleasanton, CA), first in a breathing chamber and then via facemask. Mice were allowed to breath spontaneously during the procedure. The mice were first treated with HMGB1 (2 μg/mouse, i.v.) for 16 h and then with SB1 or SB2 (0.031 or 0.061 mg/kg, i.v.). For the in vivo permeability assay, after 6 h, 1% Evans blue dye solution in normal saline was injected intravenously into each mouse. After 30 min, the mice were euthanized and the peritoneal exudates were collected by washing the cavities with normal saline (5 mL) and centrifugation of the wash solution (200 × *g*, 10 min). The absorbance of the supernatants was measured at 650 nm. The vascular permeability was expressed as microgram of dye in the peritoneal cavity per mouse, and determined using a standard curve as previously described (Jung et al., 2016). To assess the leukocyte migration, mice were euthanized after 6 h, and the peritoneal cavities were washed with 5 mL normal saline. The obtained samples of peritoneal fluids (20 μL) were mixed with 0.38 mL Turk's solution (0.01% crystal violet in 3% acetic acid) and the number of leukocytes was counted under a light microscope.

2.13. Expression of HMGB1 receptors

The expression levels of TLR2, TLR4, and RAGE receptors were determined by whole-cell ELISA, as previously described (Jung et al., 2016). Briefly, confluent monolayers of HUVECs were treated with HMGB1 (1 μg/mL) for 16 h, treated with each compound, and fixed in 1% paraformaldehyde. After three washes, specific antibodies for TLR2, TLR4, and RAGE receptors (A-9, H-80, and A-9, respectively, Santa Cruz, CA) were added and the samples were incubated for 1 h (37 °C, 5% CO_2). The cells were then washed, treated with peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 1 h, washed three times, and treated with *o*-phenylenediamine substrate (Sigma).

2.14. ELISA for phosphorylated p38 mitogen-activated protein kinase (MAPK), TNF- α, and IL-6

The activity of phosphorylated p38 MAPK was quantified in accordance with the manufacturer's instructions using a commercially available ELISA kit (Cell Signaling Technology, Danvers, MA). The concentrations of IL-6 and TNF-α in cell culture supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN). For all assays, the values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria).

2.15. Hematoxylin and eosin staining and histopathological examination

The male C57BL/6 mice were subjected to CLP and were administered SB1 or SB2 (0.061 mg/kg, i.v.) at 12 h and 50 h after CLP (n = 5). At 96 h after CLP, the mice were euthanized. To analyze the phenotypic change in the lung, samples were removed from each mouse, washed three times in PBS (pH 7.4) to remove the remaining blood, and fixed in 4% formaldehyde solution (Junsei, Tokyo, Japan) in PBS for 20 h at 4 °C. After fixation, the samples were dehydrated using an ethanol series, embedded in paraffin, sectioned into 4-μm slices, and placed on a slide. The slides were deparaffinized in a 60 °C oven, rehydrated, and stained with hematoxylin (Sigma). To remove over-staining, the slides were quickly dipped three times in 0.3% acid alcohol and counterstained with eosin (Sigma). The over-staining was then removed by washes in an ethanol series and xylene, and the samples were placed under a coverslip. Light microscopic analysis of the lung specimens was performed by a blinded observer who evaluated the pulmonary architecture, tissue edema, and infiltration of the inflammatory cells using a previously defined method (Ozdulger et al., 2003). The samples were classified into four grades: Grade 1 represented normal histopathology; Grade 2 indicated minimal neutrophil leukocyte infiltration; Grade 3 represented moderate neutrophil leukocyte infiltration, perivascular edema formation, and the partial destruction of pulmonary architecture; Grade 4 included dense neutrophil leukocyte infiltration, abscess formation, and the complete destruction of pulmonary architecture.

2.16. Statistical analysis

All the experiments were independently performed a minimum of three times. The values were expressed as the means ± standard deviation (SD). The statistical significance of differences between the test groups was evaluated by SPSS for Windows, version 16.0 (SPSS, Chicago, IL). The statistical relevance was determined by one-way analysis of variance (ANOVA) and Tukey's post-test. The values of $p < 0.05$ were considered to indicate statistical significance. The survival of CLP-induced sepsis outcomes was assessed using Kaplan–Meier analysis.

3. Results and discussion

3.1. Effects of **SB1 and SB2** on LPS and CLP-induced secretion of HMGB1

It is well established that HMGB1 is secreted by activated macrophages and necrotic cells, and functions as a “danger signal” to augment severe inflammatory response (Bae and Rezaie, 2011; El Gazzar, 2007; Mullins et al., 2004; van Beijnum et al., 2008). The levels of HMGB1 slowly increase after 8 h and are correlated with the progression of sepsis (Czura et al., 2003). Thus, we investigated the effect of **SB1** and **SB2** on the LPS-induced secretion of HMGB1 by HUVECs. Treatment with **SB1** or **SB2** inhibited the secretion of HMBG1 in a dose-dependent manner, with a minimal effective concentration of 20 μM (Fig. 1A). To confirm this effect in vivo, we evaluated the ability of **SB1** and **SB2** to inhibit the release of HMGB1 using a mouse model of CLP-induced sepsis. Treatment with **SB1** or **SB2** resulted in a significant reduction in HMGB1 secretion in this model (Fig. 1B). As the average circulating blood volume for mice is 72 mL/kg (Diehl et al., 2001) and the average weight of mouse used was 27 g, the average blood volume was 2 mL. Hence, the amount of injected **SB1** or **SB2** (0.031 or 0.061 mg/kg) yielded a maximum concentration of 20 or 40 μM in the peripheral blood.

Previous studies indicated that hyperacetylation of HMGB1 affected its ability to bind DNA and redirected it toward the cytoplasm (Bonaldi et al., 2003) and that hyperacetylation on the serine residues of HMGB1 blocked its nuclear import and promoted cytoplasmic secretion (Youn and Shin, 2006). In addition, the activation of Sirtuin 1 (SIRT1) plays a pivotal role in the deacetylation of HMGB1; HMGB1 is a novel deacetylation target of SIRT1 (Rabadi et al., 2015). Therefore, to determine the effects of **SB1** and **SB2** on the induction of SIRT1 expression, we performed a western blot analysis. As shown in Fig. 1C, the expression of SIRT1 was apparent after 4 h of incubation; it peaked after 6 h, was maintained until 8 h, and disappeared at 12 h. In addition, to define the molecular mechanism by which **SB1** or **SB2** suppressed the secretion of HMGB1 by LPS, we determined the effects of **SB1** and **SB2** on the deacetylation of HMGB1 and on the induction of SIRT1 expression. As shown in Fig. 1D, stimulation with LPS increased the acetylation of

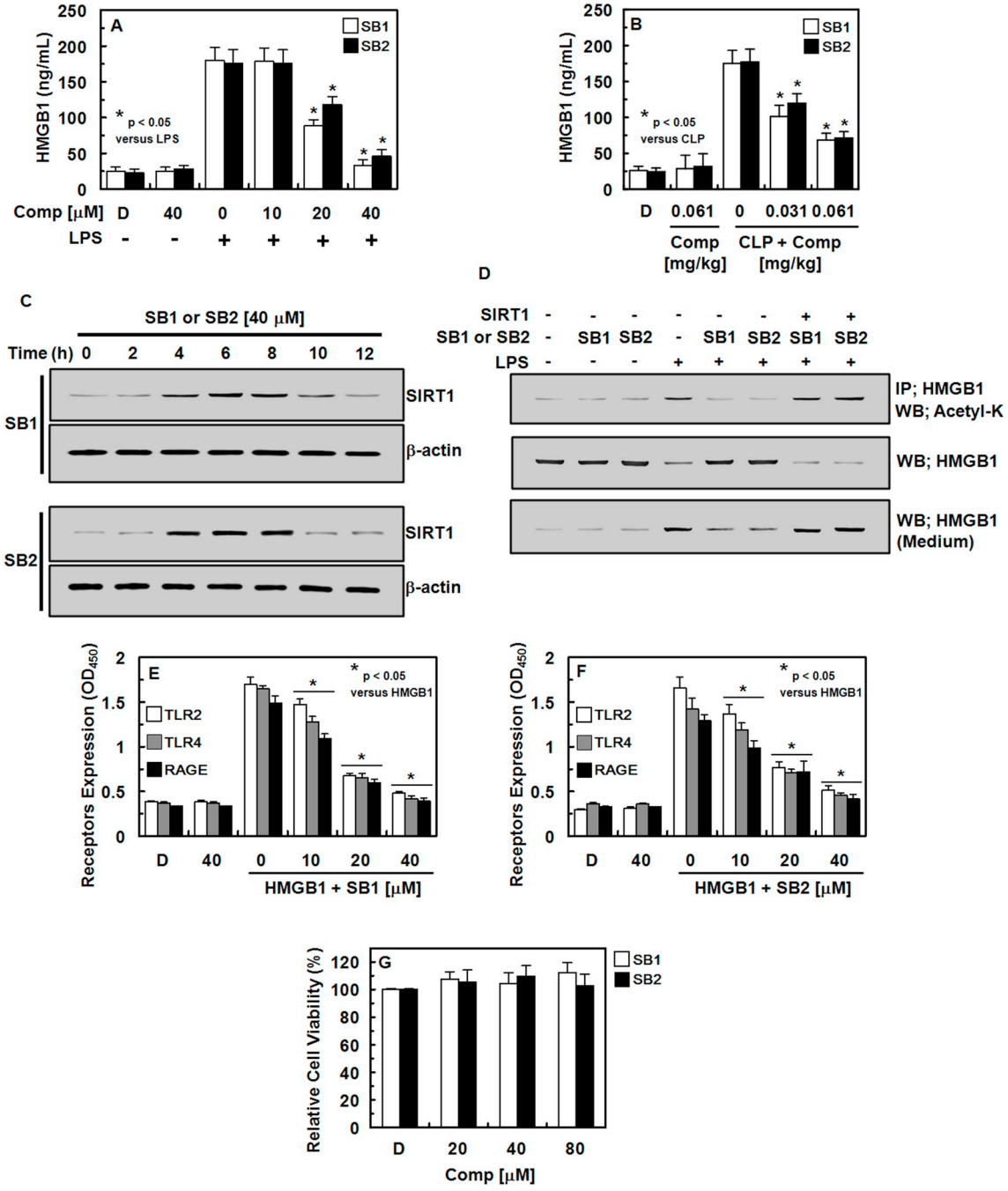

Fig. 1. Effects of SB1 and SB2 on the release of high mobility group box-1 protein (HMGB1) and expression of HMGB1 receptors. (A) After stimulation with lipopolysaccharide (LPS; 100 ng/mL, 16 h), primary human umbilical vein endothelial cells (HUVECs) were treated with the indicated concentrations of **SB1** or **SB2** for 6 h and the released HMGB1 was measured by ELISA. (B) Twelve hours after cecal ligation and puncture (CLP), male C57BL/6 mice (n = 5) were intravenously administered the indicated amount of **SB1** or **SB2**, and euthanized after 24 h of CLP. The serum HMGB1 levels were measured by ELISA. (C, D) Cells were treated with **SB1** or **SB2** (40 μM) for 0, 2, 4, 6, 8, 10, and 12 h. After incubation for the indicated time, the cells were lysed and analyzed via western blotting to measure the expression levels of SIRT1 (C). Effect of **SB1** or **SB2** on the acetylation of HMGB1 and the SIRT1 expression in HUVECs. Cells were treated with LPS (100 ng/mL) with or without **SB1** or **SB2** (40 μM). Alternatively, cells were treated with the SIRT1 inhibitor (sirtinol, Srtnl, 10 mM) for 1 h prior to **SB1** or **SB2** treatment. After incubation for 6 h, the cells were lysed for immunoprecipitation. Cell lysates were subjected to immunoprecipitation with anti-HMGB1 antibody and HMGB1 acetylation and total HMGB1 protein level were measured by immunoblot analysis using anti-acetyl-lysine (K) or anti-HMGB1 antibodies, respectively (D, rows 1 and 2). After incubation for 16 h, equal volumes of medium were collected and the released HMGB1 was detected by western blotting (D, row 3). (E, F) Confluent HUVECs were activated with HMGB1 (1 μg/mL, 16 h) and then incubated with **SB1** (E) or **SB2** (F) for 6 h. Expression levels of TLR2 (white bar), TLR4 (gray bar), or RAGE (black bar) were determined by cell-based ELISA. (G) The effect of **SB1** or **SB2** on cellular viability was measured by MTT assay. The results shown are the means ± SD from three separate experiments conducted in triplicate on different days. D = 0.2% DMSO is the vehicle control. *$p < 0.05$ versus LPS alone (A), CLP alone (B), or HMGB1 alone (E, F).

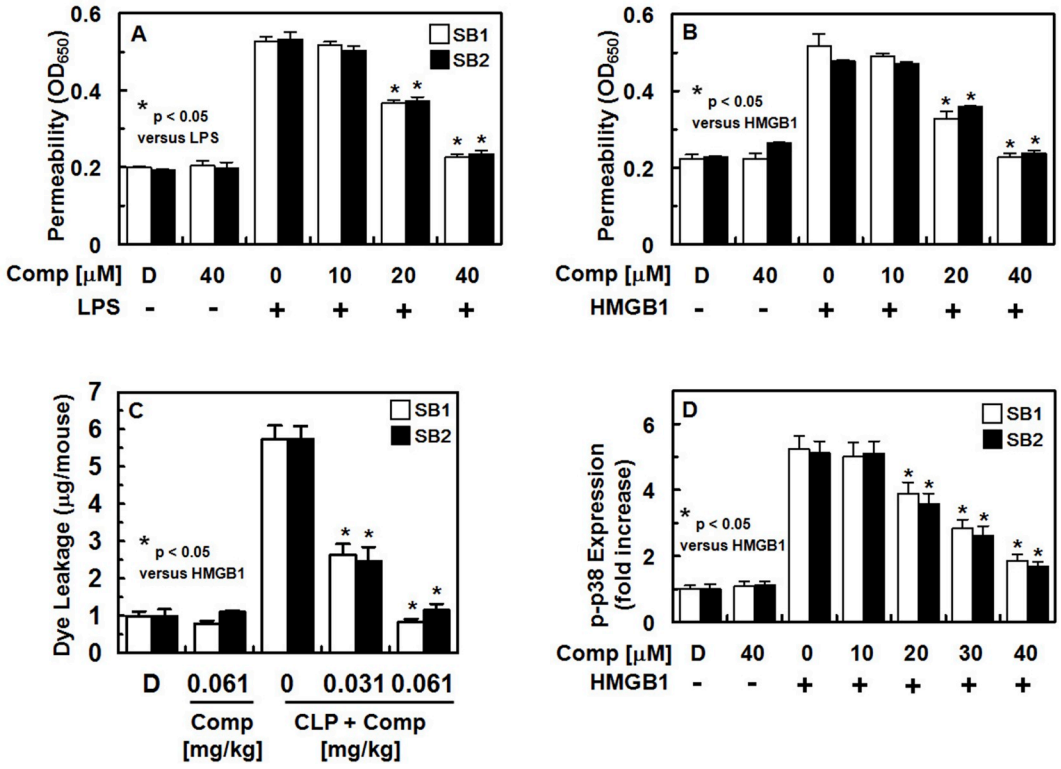

Fig. 2. Effects of SB1 and SB2 on high mobility group box-1 protein (HMGB1)-mediated permeability, *in vitro* and *in vivo*. (A, B) Effects of treatment with different concentrations of **SB1** or **SB2** for 6 h on barrier disruption caused by lipopolysaccharide (LPS; 100 ng/mL, 4 h; A) or HMGB1 (1 μg/mL, 16 h; B) were monitored by the measurement of the flux of Evans blue-bound albumin across the primary human umbilical vein endothelial cells (HUVECs). (C) The effects of **SB1** and **SB2** on HMGB1-induced (2 μg/mouse, i.v.) vascular permeability in mice were examined through the measurement of Evans blue dye in peritoneal washings (expressed as μg/mouse, n = 5). (D) HUVECs were activated with HMGB1 (1 μg/mL, 16 h), followed by treatment with different concentrations of **SB1** or **SB2** for 6 h. Effects of **SB1** and **SB2** on the HMGB1-mediated expression of phospho-p38 were determined by ELISA. Results are expressed as the means ± SD of three separate experiments on different days. D = 0.2% DMSO is the vehicle control. $^*p < 0.05$ versus LPS (A) or HMGB1 (B, C, D).

HMGB1; this change was mostly reduced by the addition of **SB1** and **SB2**. To confirm that SIRT1 activity was responsible for the inhibition of HMGB1 release via the deacetylation of HMGB1 in LPS-activated HUVECs, we investigated the effect of sirtinol, a SIRT1 inhibitor, on the release of HMGB1. We observed that treatment with sirtinol clearly reversed the effect of **SB1** and **SB2** (Fig. 1D) and significantly increased both the acetylation and secretion of HMGB1. Therefore, these results suggested that **SB1** and **SB2** significantly reduced HMGB1 release in LPS-activated HUVECs via the SIRT1-mediated deacetylation of HMGB1.

Next, we determined whether **SB1** and **SB2** inhibited the expression of HMGB1 receptors, such as TLR2, TLR4, and RAGE, in HUVECs. The data showed that HMGB1 increased the expression of each receptor and **SB1** and **SB2** diminished this increase in expression (Fig. 1E and F). Additionally, cell viability assays were performed to probe the toxicity of **SB1** and **SB2** in HUVECs after 48 h of treatment. At the tested concentrations (up to 80 μM), **SB1** and **SB2** did not affect cell viability (Fig. 1G). Collectively, these results indicated that administration of **SB1** and **SB2** might be a viable early intervention to prevent the release of HMGB1 and inhibit the subsequent progression to severe sepsis and septic shock.

3.2. Effect of *SB1* and *SB2* on HMGB1-mediated vascular barrier disruption

As HMGB1 and LPS are known to disrupt vascular barrier integrity (Lee et al., 2014b), a vascular permeability assay was performed to evaluate the effects of **SB1** and **SB2** on the maintenance of barrier integrity in HUVECs. HUVECs were treated with **SB1** or **SB2** for 6 h following the activation with LPS (Fig. 2A, 100 ng/mL) or HMGB1 (Fig. 2B, 1 μg/mL). We observed that **SB1** and **SB2** inhibited the LPS- and HMGB1-mediated hyperpermeability in a dose-dependent manner (Fig. 2A and B). To verify these results, the effects of **SB1** and **SB2** on the vascular permeability were assessed in vivo. We observed that **SB1** and **SB2** markedly inhibited peritoneal dye leakage through the action of HMGB1 (Fig. 2C). As the vascular disruptive responses caused by HMGB1 occur through various signaling pathways (the activation of ERK 1/2 and p38 MAPK downstream of TLR2/4, and the Ras/p38 pathway downstream of RAGE (Palumbo et al., 2007; Qin et al., 2009; Sun et al., 2009b)), we next determined whether the activation of p38, a common signaling target of HMGB1 receptor, was affected by **SB1** and **SB2**. To achieve this, HUVECs were treated with **SB1** or **SB2** following activation with HMGB1. As shown in Fig. 2D, HMGB1 upregulated the expression of phosphorylated p38; this upregulation was clearly reduced by treatment with **SB1** and **SB2**. The reduction in HMGB1-induced permeability and p38 activation indicated the promising role of **SB1** and **SB2** as antisepsis drugs.

3.3. Effects of *SB1* or *SB2* on HMGB1-mediated adhesion, and migration of human neutrophils

The adhesion and migration of immune cells toward the endothelium at the site of vascular inflammation are involved in vascular inflammatory diseases (Frenette and Wagner, 1996; Ulbrich et al., 2003). Therefore, we examined the inhibitory effects of **SB1** and **SB2** on the adhesion and migration of human neutrophils in HMGB1-activated

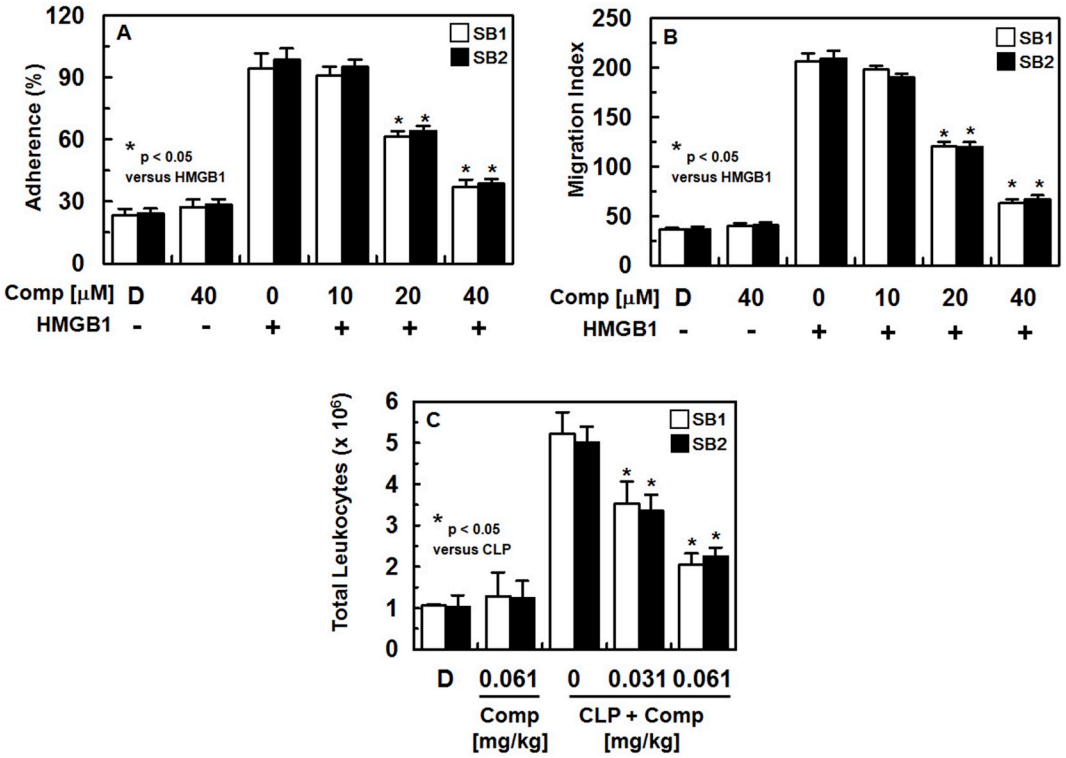

Fig. 3. Effects of SB1 and SB2 on high mobility group box-1 protein (HMGB1)-mediated pro-inflammatory response. Primary human umbilical vein endothelial cells (HUVECs) were stimulated with HMGB1 (1 μg/mL) for 16 h and then treated with **SB1** or **SB2** for 6 h. HMGB1-mediated (A) adherence of human neutrophils to HUVEC monolayers, and (B) migration of neutrophils through HUVEC monolayers were analyzed. (C) The effects of treatment with **SB1** or **SB2** on HMGB1-induced (2 μg/mouse, i.v.) leukocyte migration into the peritoneal cavities of mice were analyzed. All the results indicate the mean ± SD of three separate experiments on different days. D = 0.2% DMSO is the vehicle control. *$p < 0.05$ versus HMGB1 (A, B) or cecal ligation and puncture (CLP) (C).

HUVECs. As shown in Fig. 3A and B, **SB1** and **SB2** reduced the adherence of human neutrophils to HUVECs and their subsequent migration. These results were corroborated in vivo by the inhibition of HMGB1-induced migration of leukocytes in the peritoneal space (Fig. 3C). Thus, our results suggested that **SB1** and **SB2** inhibited the adhesion and migration of leukocytes to the inflamed endothelium.

3.4. Effects of *SB1* or *SB2* on HMGB1-stimulated activation of NF-κB/ERK and production of IL-6/TNF- α

HMGB1 is known to contribute to pathophysiological systemic inflammation by upregulation of inflammatory cytokines, such as TNF-α and IL-6, either individually or in combination with other pro-inflammatory cytokines (Erlandsson Harris and Andersson, 2004; Jung et al., 2016; Lee et al., 2014a). As mentioned above, HMGB1 stimulated the release of inflammatory cytokines through various signaling pathways [10,32,35], including ERK 1/2, and ultimately resulted in the activation of NK-κB. Thus, in order to determine the mechanism of inhibition of HMGB1-mediated septic response by **SB1** and **SB2**, their effects on HMGB1-induced production of TNF-α and IL-6, or on the activation of NF-κB and ERK 1/2, were evaluated. HUVECs were activated with HMGB1 for 16 h, followed by incubation with **SB1** or **SB2** for 6 h. Our results showed that HMGB1 enhanced the production of TNF-α and IL-6 and the activation of NF-κB and ERK 1/2, and that these increases were significantly reduced by treatment with **SB1** and **SB2** (Fig. 4A and B). To confirm this effect in vivo, we evaluated the ability of **SB1** and **SB2** to inhibit the production of TNF-α and IL-6 in CLP. Treatment with **SB1** or **SB2** resulted in a significant reduction in the production of TNF-α and IL-6 (Fig. 4C and D). Furthermore, HMGB1 induced an increase in the expression of p65 NF-κB in the nucleus, whereas this was not elevated under normal conditions. Treatment with **SB1** and **SB2** decreased HMGB1-induced expression of p65 NF-κB in the nucleus (Fig. 4E). And, treatment with HMGB1 resulted in increased activation of ERK 1/2, and these increases significantly reduced after treatment with **SB1** and **SB2** (Fig. 4F).

3.5. Protective effect of *SB1* and *SB2* in CLP-induced septic mice

To evaluate whether the suppression of HMGB1 secretion and HMGB1-mediated septic response by **SB1** and **SB2** also influenced the survival rate of CLP-induced septic mice, **SB1** or **SB2** was administered to mice after CLP surgery. A single administration of **SB1** or **SB2** (0.061 mg/kg, 12 h after CLP) did not prevent CLP-induced death (data not shown). Thus, we administered two equal doses of **SB1** or **SB2**, one at 12 h after CLP and the other at 50 h after CLP, and observed that **SB1** and **SB2** increased the survival rate of septic mice, according to the Kaplan–Meier survival analysis ($p < 0.00001$, Fig. 5A–C). To compared the beneficial effects of **SB1** or **SB2**, sulforaphane (SFN) was used as a positive control (Lee et al., 2017) (Fig. 5D). The marked improvement in survival rate achieved by the administration of **SB1** or **SB2** suggested that **SB1** and **SB2** might be used for the treatment of severe vascular inflammatory diseases, such as sepsis and septic shock.

3.6. Protective effect of *SB1* and *SB2* in CLP-induced pulmonary injury

We determined the effects of **SB1** and **SB2** on CLP-induced pulmonary injury to confirm the protective activity of **SB1** and **SB2** in CLP-induced death. The CLP surgery resulted in interstitial edema owing to massive infiltration of inflammatory cells in the interstitium and alveolar spaces and the pulmonary architecture was severely impaired

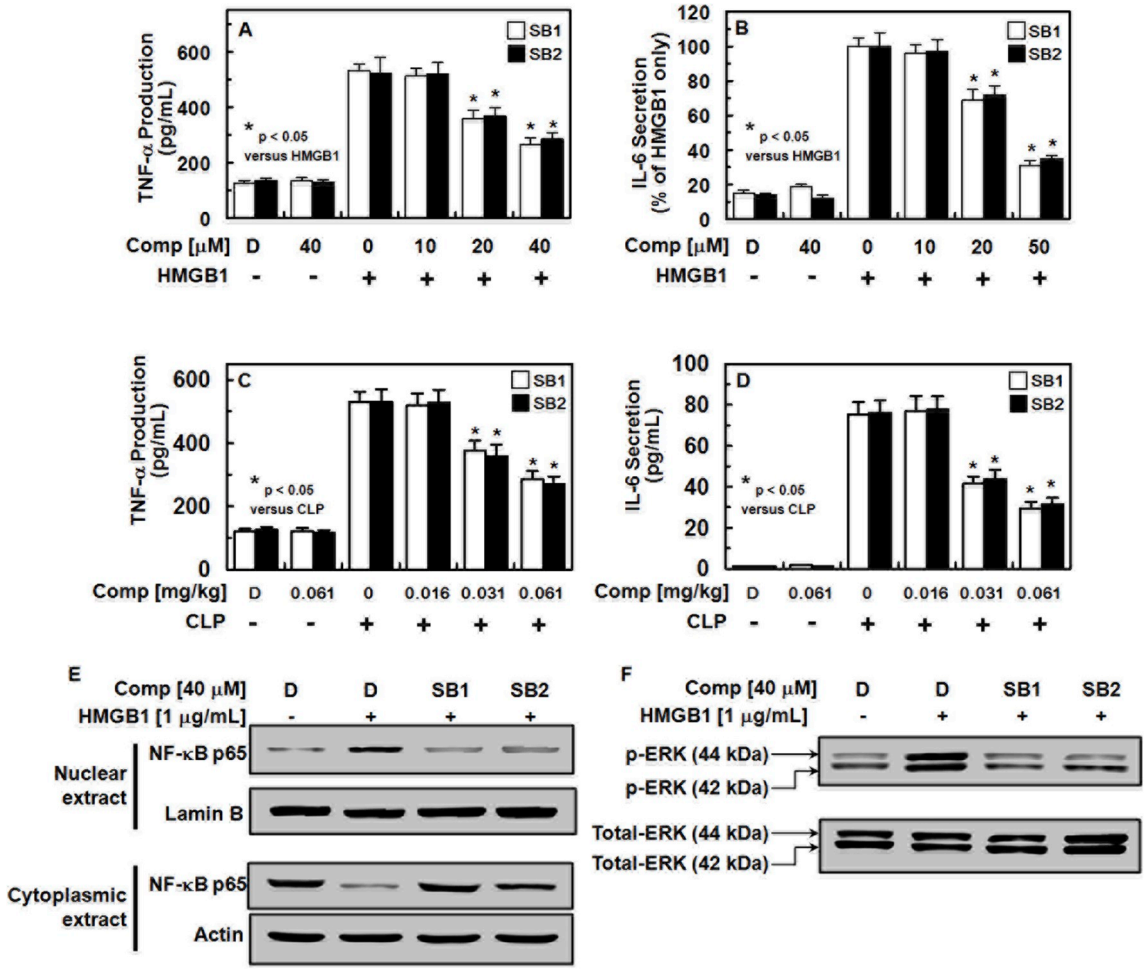

Fig. 4. Effects of SB1 and SB2 on high mobility group box-1 protein (HMGB1)-stimulated production of IL-6/TNF-α and activation of NF-κB/ERK. (A,B) Primary human umbilical vein endothelial cells (HUVECs) were stimulated with HMGB1 (1 μg/mL) for 16 h and then treated with **SB1** or **SB2** for 6 h (C,D) Twelve hours after cecal ligation and puncture (CLP), male C57BL/6 mice (n = 5) were intravenously administered the indicated amount of **SB1** or **SB2**, and euthanized after 24 h of CLP. HMGB1 (A,B)- or CLP- (C,D) mediated production of TNF-α (A, C) or IL-6 (B, D) was analyzed. (E) Expression levels of NF-κB in nuclear or cytoplasmic extracts were evaluated by western blotting. β-actin and lamin B were used as loading controls for cytoplasmic and nuclear extracts, respectively. (F) The levels of phospho-ERK1/2 and total ERK1/2 in HUVECs were analyzed by western blotting. Results are expressed as the means ± SD of three separate experiments on different days. D = 0.2% DMSO is the vehicle control. *$p < 0.05$ versus HMGB1 (A,B) or CLP (C,D).

(Fig. 5F). These disordered morphological changes were reduced in the CLP mice treated with **SB1** or **SB2** (Fig. 5E and F).

We evaluated the protective effect of **SB1** and **SB2** against vessel barrier integrity. Under physiological conditions, the vascular endothelium plays a central role in maintaining and controlling the vascular integrity in response to the extracellular environment and is, therefore, a primary target of sepsis induced damage (Bogatcheva and Verin, 2008). Because all blood vessels are lined with endothelial cells, the presence of vascular leak and tissue edema in sepsis suggests endothelial dysfunction. The breakdown of vascular integrity and endothelial dysfunction resulting from septic attack may result in hyperpermeability (Bogatcheva and Verin, 2008). It is known that continuous, subcutaneous, and body-cavity edema typically develop in septic patients; this is suggestive of a comprehensive increase in vascular permeability (Bogatcheva and Verin, 2008). Therefore, the restoration of vascular integrity from disruptive response by inflammatory stimuli and the maintenance of vascular homeostasis should be the primary strategy against sepsis, which will result in spontaneous diuresis with a reduction in edema. The results of this study suggested that the antiseptic effects of **SB1** or **SB2** occurred through the inhibition of HMGB1 release and HMGB1-mediated hyperpermeability.

The molecular mechanism underlying the anti-inflammatory effects of **SB1** or **SB2** against HMGB1-mediated septic response might be facilitated by the suppression of HMGB1 release via the SIRT1-induced deacetylation of HMGB1 (Fig. 1A–D), the expression of HMGB1 receptors, such as TLR2, TLR4, and RAGE (Fig. 1E), and HMGB1-mediated hyperpermeability (Fig. 2B and C) via the suppression of p38 activation (Fig. 2D). The mechanism underlying the anti-inflammatory effects of **SB1** and **SB2** was the downregulated production of inflammatory cytokines, TNF-α and IL-6 (Fig. 4A–D), and the activation of the inflammatory transcriptional factors, NF-κB and ERK1/2 (Fig. 4E and F). **SB1** and **SB2** also inhibited the translocation of NF-κB from the cytosol to the nucleus (Fig. 4E).

Collectively, the results of this study demonstrated that **SB1** and **SB2** reduced the release of HMGB1 in LPS-activated HUVECs via the SIRT1-mediated deacetylation of HMGB1, and suppressed the CLP-mediated release of HMGB1, expression of HMGB1 receptors, and HMGB1-mediated barrier disruption by increasing the barrier integrity. Furthermore, **SB1** and **SB2** reduced the adhesion and migration of leukocytes toward HUVECs. The barrier protective effects of **SB1** and **SB2** were confirmed in a mouse model, in which treatment with **SB1** or **SB2** reduced the CLP-induced mortality and pulmonary injury. Our findings indicate that **SB1** and **SB2** might be potential candidates for use in the treatment of severe vascular inflammatory diseases, such as sepsis and septic shock.

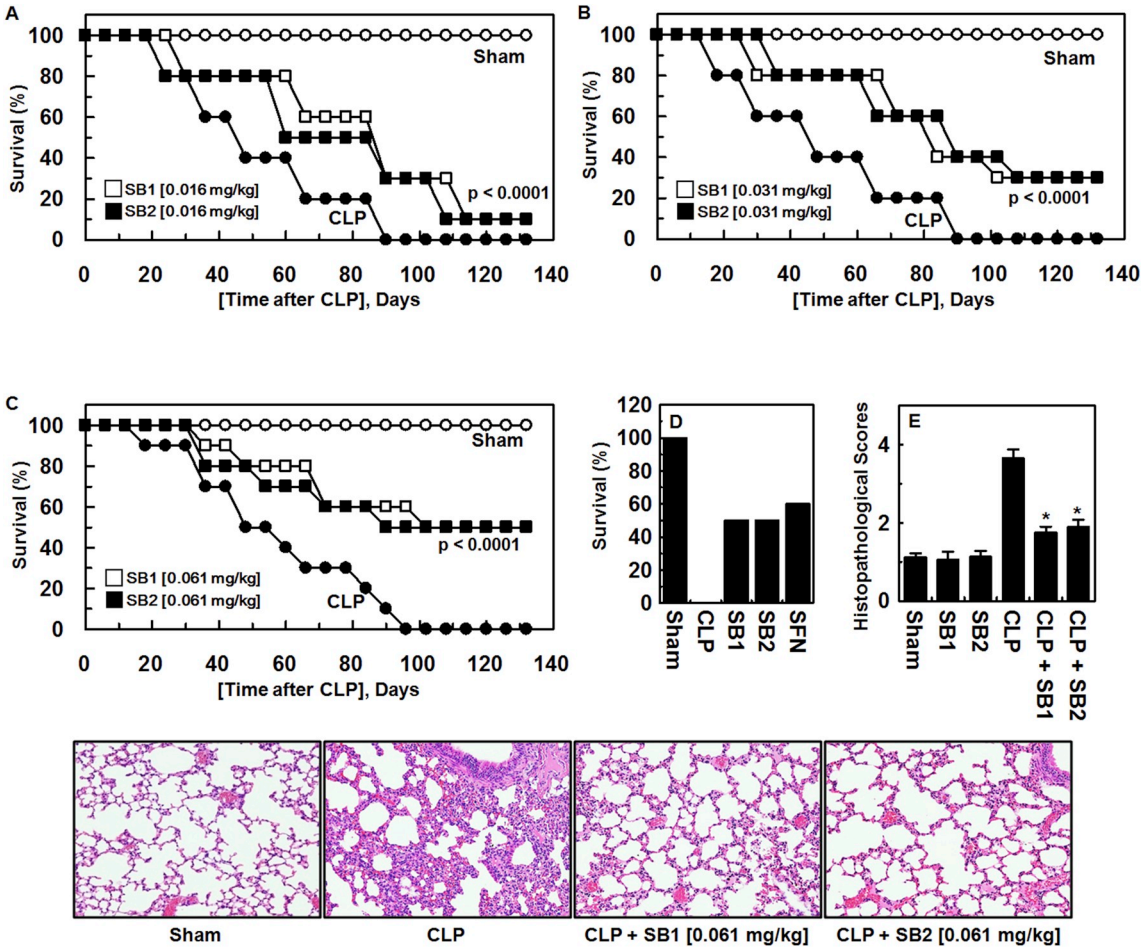

Fig. 5. Effects of SB1 and SB2 on lethality or tissue injury after cecal ligation and puncture (CLP). (A–C) Male C57BL/6 mice (n = 20) were administered **SB1** (□) or **SB2** (■) at 0.016 mg/kg (A), 0.031 mg/kg (B), or 0.061 mg/kg (C) at 12 h and 50 h after CLP. Animal survival was monitored every 12 h for 132 h after CLP. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline (n = 20). (D) Male C57BL/6 mice (n = 20) were administered SB1 (0.061 mg/kg), SB2 (0.061 mg/kg), or SFN (0.39 mg/kg) at 12 h and 50 h after CLP. Final survival rate at 132 h after CLP was shown. Kaplan–Meier survival analysis was used to determine the overall survival rates versus CLP treated mice. (E) Male C57BL/6 mice were subjected to CLP, administered **SB1** or **SB2** intravenously at 12 h and 50 h after CLP (n = 5), and euthanized at 96 h after CLP. Histopathological scores for the lung tissue were recorded as described in methods section. (F) Photomicrographs of lung tissues (hematoxylin and eosin staining, × 200). Illustrations are representative images from three independent experiments conducted on different days with similar results. All results indicate the mean ± SD of three separate experiments conducted on different days with similar results. *$p < 0.05$ versus CLP.

Conflicts of interest

The authors have no conflicts of interest to declare.

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