Supporting Information

Dual Functioned PEGylated phospholipid micelles containing cationic antimicrobial decapeptide for treating sepsis

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Supplementary Materials and Methods

Reagents: Bacterial LPS (serotype: 0111:B4, L5293), antibiotics (penicillin G, streptomycin, vancomycin, and gentamicin), a 37-mer peptide (LL-37; L^{1}LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES^{37}), and lactoferricin B were purchased from Sigma (St. Louis, MO). Anti-OCLN polyclonal antibody (71-1500) was purchased from Thermo Fisher Scientific, and anti-mouse CD31 antibody (553369) was purchased from BD Falcon. Anti-phosphorylated Tyr antibody (#9411) and anti-phosphorylated Thr antibody (#9381) were purchased from Cell Signaling Technology, and anti-phosphorylated Ser antibody (ab9332) and recombinant human OCLN (ab114189) were purchased from Abcam. KSLW (KKVVFWVKFK) with a molecular weight (MW) of 1,308 Da was obtained from Peptron (Daejeon, Korea). Monomethoxy PEG–ALD with a MW 5,000 Da and N-(3'-oxopropylpolyoxyethylene oxycarbonyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE–PEG–ALD) with a MW 4,042 Da (PEG MW, 3,400 Da) were obtained from NOF Corporation (Tokyo, Japan). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise indicated, and were used as received.

Preparation and separation of PEG5K–KSLW: KSLW was PEGylated in the presence of sodium cyanoborohydride ($NaCNBH_3$) under mild acidic conditions, as described previously [1]. PEGylation was performed by adding 1 mL KSLW solution (10 mg/mL in water) to 1 mL PEG–ALD in 0.1 M acetate buffer containing 40 mM $NaCNBH_3$ (pH 5.5) at a molar ratio of 1:1 (KSLW:PEG–ALD). The reaction was allowed to continue for 18 h at 4°C. PEG5K–KSLW in the reaction mixture was separated by performing RP–HPLC by using Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA). Separation was performed using Gemini C-18 column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) by performing

linear gradient elution with 0.1% trifluoroacetic acid (TFA) in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B) as the mobile phase. The gradient elution was performed as follows: 0–15 min with 20–50% mobile phase B, 15–16 min with 50–20% mobile phase B, and 16–20 min with 20% mobile phase B. Flow rate was maintained at 1.0 mL/min, and UV absorbance was measured at 215 nm. PEG5K–KSLW fractions eluted from 11.8 to 12.8 min were collected and were concentrated using Amicon centrifugal filters (MW cut-off, 3 kDa; Millipore Corporation, Billerica, MA).

Preparation and separation of PLM–KSLW: KSLW-conjugated DSPE–PEG micelles (PLM–KSLW) were synthesized by performing a reductive amination reaction between the ALD group of DSPE–PEG–ALD and the N-terminal amine of KSLW in the presence of $NaCNBH_3$ under mild acidic conditions. The reaction was performed by adding 1 mL KSLW solution (10 mg/mL in water) to 2 mL DSPE–PEG solution (15.5 mg/mL) in 0.1 M acetate buffer containing 33 mM $NaCNBH_3$ (pH 5.5) at a molar ratio of 1:1 (KSLW:DSPE–PEG–ALD). The conjugation reaction was performed for 18 h at 10°C. After reaction completion, the reaction mixture was dialyzed against deionized water by using a dialysis membrane (MW cut-off, 10 kDa; Spectrum Laboratories, Laguna Hills, CA) for 72 h, with frequent exchange of fresh water to remove unreacted KSLW and other reagents. The final product (PLM–KSLW) was obtained by performing dialysis against 10 mM phosphate-buffered saline (PBS) at pH 7.4 for 3 h. The loading capacity was determined by quantitation of unreacted KSLW present in the filtrate followed by ultrafiltration (MW cut-off, 10 kDa) using reversed-phase HPLC method. These results were compared with KSLW concentration in the final product determined by micro BCA assay (Pierce, Rockford, IL, USA).

MALDI–TOF MS of PEG5K–KSLW and PLM–KSLW: Molecular masses of PEG5K–KSLW and PLM–KSLW were determined by performing MALDI–TOF MS with Microflex MALDI–TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with a 337-nm nitrogen laser. Mass spectra were obtained in a linear and positive-ion mode, with a least 100 shots per spectrum. A saturated solution of sinapinic acid in acetonitrile:water (50:50, v:v) containing 0.1% TFA was used as a matrix solution. Each analyte was mixed with the matrix solution at a ratio of 1:1 (analyte:matrix, v:v), and 1 μL of the analyte–matrix solution was deposited onto the sample plate and was air dried.

DLS assay of PEG5K–KSLW and PLM–KSLW: The average hydrodynamic diameters and %Pd values of PEG5K–KSLW and PLM–KSLW were measured by performing DLS with DelsaMax PRO (Beckman Coulter, Brea, CA). Samples for DLS measurement were filtered using a 0.22-μm membrane after adjusting their concentration to 2 mg/mL in 10 mM PBS based on PEG and DSPE–PEG concentrations. Measurements were performed by repeating three times, with 10 subruns at 20°C. Data were acquired using DelsaMax Analysis Software version 1.0.

Pharmacokinetic analysis: To investigate the pharmacokinetics parameters and terminal elimination half-life ($T_{1/2}$, λ_z) time of KSLW, PEG5K–KSLW, or PLM–KSLW, each construct was dissolved in saline and was intravenously injected into male ICR mice at a dose of 500 μg/kg. Approximately 50 μL blood was obtained from the tail vein of each mouse at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, and 32 h before and after the intravenous injection and was immediately placed in heparin-coated glass tubes. Plasma samples

were obtained by centrifuging the blood samples at 1500 × *g* for 10 min. Mouse plasma-coated plates were incubated with 1 μM OCLN for 1 h, and binding affinities of KSLW, PEG5K–KSLW, and PLM–KSLW toward OCLN were determined by performing ELISA. Colorimetric analysis was performed by measuring the absorbance at 492 nm. Experimental data and pharmacokinetic parameters were calculated using a non-compartmental model with WinNonlin (Version 2.1, Scientific Consulting, KY, USA). Results are expressed as a relative value compared to the quantified value of negative control not treated with each construct. All measurements were performed in triplicate wells.

Attachment of HUVECs to KSLW, PEG5K–KSLW, or PLM–KSLW: The attachment of HUVECs on each construct was determined by CD31 ELISA. KSLW, PEG5K–KSLW, or PLM–KSLW (2 mM) were coated on non-cell adherent plates and HUVECs were seeded and incubated for 12 h. After washing with PBS, 100 mL of rabbit antihuman CD31 antibody was added. After 1 h (37°C, 5 % CO_2), plates were washed three times with PBS, followed by addition of 100 μL of 1:2000 peroxidase-conjugated antirabbit IgG antibodies (Sigma, Saint Louis, MO, USA) for 1 h. After washing, plates were developed using *o*-phenylenediamine substrate (Sigma). Colorimetric analysis was performed by measuring absorbance at 490 nm. All measurements were performed in triplicate wells.

Animals and husbandry: Male C57BL/6 mice (age, 6–7 weeks; average weight, 26 g) were purchased from Orient Bio Co. (Sungnam, Kyungki-Do, Republic of Korea) and were used after a 12-day acclimatization period. The mice were housed in polycarbonate cages (n = 5/cage) under controlled temperature (20–25°C) and humidity (40–45%) and under 12-/12-h light/dark cycle. The mice were fed a normal rodent pellet diet

and were supplied water *ad libitum*. All the mice 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).

Cell culture: Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and were maintained as described previously [2-4]. HUVECs were used in cell culture at passages 3-5.

Cecal ligation and puncture: For inducing sepsis, male mice were anesthetized using 2% isoflurane (Forane; JW Pharmaceutical, Republic of Korea) in oxygen that was delivered using a small rodent gas anesthesia machine (RC2; Vetequip, Pleasanton, CA) first in a breathing chamber and then through a facemask. The mice were allowed to breath spontaneously during the procedure. The mouse model of CLP-induced sepsis was established, as described previously [2, 5]. In brief, a 2-cm midline incision was made to expose the cecum and the adjoining intestine. The cecum was tightly ligated using a 3.0 silk suture at 5.0 mm from the cecal tip and was punctured once using a 22-gauge needle for inducing high-grade sepsis [6]. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and was placed back into the peritoneal cavity. The laparotomy site was then sutured using a 4.0 silk suture. In the sham-operated control mice, the cecum was exposed but was not ligated or punctured and was placed back into the abdominal cavity. This protocol was approved by the Animal Care Committee at Kyungpook National University before conducting the study (IRB No. KNU 2016-54).

Mouse model of LPS-induced sepsis: The mice were intravenously injected with 30 mg/kg LPS and were intravenously treated with KSLW, PEG5K–KSLW, or PLM–KSLW. Lethal dosage was determined by

injecting the mice with increasing concentrations of LPS and by determining mortality over a 24-h period [7]. Animal experiments were performed according to the protocols approved by Animal Experimentation Ethics Committee in the Animal Care Committee at the Kyungpook National University (IRB No. KNU 2016-54).

Antimicrobial susceptibility assay: The antimicrobial activities of KSLW, PEG5K–KSLW, and PLM–KSLW were determined by evaluating bacterial growth inhibition and bactericidal activity. Microtiter broth dilution method was used [8]. The antimicrobial activities of KSLW, PEG5K–KSLW, and PLM–KSLW were examined using gram-negative *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, and *Pseudomonas aeruginosa* ATCC 10145 and gram-positive *Staphylococcus aureus* ATCC 25923, *Streptococcus faecium* ATCC 8043, and *Enterococcus faecalis* ATCC 29212. Briefly, bacterial suspensions (5×10^5 cells/mL) were incubated with two-fold dilutions of the constructs in 96-well nonbinding surface plates (Corning) for 24 h at 37°C. Minimal inhibitory concentration was defined as the lowest construct concentration that resulted in no visible bacterial growth. To confirm whether the constructs killed the test bacteria, minimal bactericidal concentration (MBC) was determined by adding 30 μL resazurin dye (0.01%, w/v) to each well. The plates were incubated at 37°C for additional 18 h. Wells with a blue coloration indicate the presence of dead bacteria, and wells with a pink coloration indicate the presence of live bacteria. The MBC value was defined as the lowest concentration of the constructs that resulted in blue coloration in the wells. The assay was performed using Mueller–Hinton broth and was repeated four times.

Hematoxylin and eosin staining and histopathological examination: Male C57BL/6 mice were operated by performing CLP and were intravenously injected with KSLW, PEG5K–KSLW, or PLM–KSLW (500 μg/kg) at 12 h after CLP (n = 5). The mice were euthanized at 96 h after CLP. To analyze phenotypic changes in the lung, kidney, and liver of CLP-operated mice, lung samples were obtained from each mouse, washed three times with PBS (pH 7.4) to remove residual blood, and fixed in 4% formaldehyde solution (Junsei, Tokyo, Japan) in PBS (pH 7.4) for 20 h at 4°C. After fixation, the samples were dehydrated using an ethanol series, embedded in paraffin, sectioned into 4-μm-thick sections, and placed on a slide. The slides were deparaffinized in a 60°C oven, rehydrated, and stained with hematoxylin (Sigma). To remove overstaining, the slides were rapidly dipped three times in 0.3% acid alcohol and were counterstained with eosin (Sigma). The slides were then washed in an ethanol series and xylene and were covered with a coverslip. The lung specimens were examined under a light microscope in a blinded manner to evaluate pulmonary architecture, tissue edema, and infiltration of inflammatory cells, as described previously [9].

In vivo permeability assays: CLP-operated mice were intravenously injected with KSLW, PEG5K–KSLW, or PLM–KSLW. After 6 h, the mice were intravenously injected with 1% Evans blue dye solution in normal saline. After 30 min, the mice were killed. Their peritoneal exudates were collected after washing with normal saline (5 mL) and were centrifuged at 200 × g for 10 min. Absorbance of the supernatant was read at 650 nm. Vascular permeability was expressed in terms of dye (μg/mouse) that passed into the peritoneal cavity based on the standard curve of Evans blue dye described previously [10].

In vitro permeability assay: To spectrophotometrically quantify the changes in endothelial cell permeability in response to the treatment with increasing concentrations of each construct, the flux of Evans blue-bound albumin across functional cell monolayers was measured using a modified two-compartment chamber model, as described previously [11]. HUVECs were plated (5×10^4/well) in 12-mm-diameter Transwells with a pore size of 3 μm for 3 days. Confluent monolayers of HUVECs were exposed to LPS (1 μg/mL) for 4 h before treatment with KSLW, PEG5K–KSLW, or PLM–KSLW (up to 5 μM). The Transwell inserts were washed with TBS (pH 7.4) and treated with Evans blue dye (0.5 mL; 0.67 mg/mL) diluted in growth medium containing 4% BSA. Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with the medium containing Evans blue dye and BSA. After 10 min, optical density of the sample in the lower chamber was measured at 650 nm.

Expression and purification of OCLN: Plasmids expressing full-length OCLN and OCLN extracellular domain B or D were transformed into *Escherichia coli* expression strain BL21 (DE3). The cells were grown at 37°C until the OD_{600} of LB medium containing 50 μg/mL kanamycin reached 0.5. Protein expression was induced by treating the cells overnight with 0.1 M IPTG at 30°C. After induction, the cells were harvested by centrifugation. Cell pellets obtained were lysed by sonication in lysis buffer (50 mM Tris-HCl [pH 6.8], 100 mM NaCl, 1% Triton X-100, 1 mM EDTA [pH 8.0], 0.5 mM DTT, and protease inhibitor cocktail [1:1000 dilution]). After centrifugation (13000 x rpm for 20 min at 4°C) to remove bacterial debris, the bacterial lysate was affinity purified using His-tagged Purification Miniprep Kit (635710; Clontech, USA).

ELISA to determine binding affinity toward OCLN: To evaluate the binding affinities of KSLW, PEG5K–KSLW, and PLM–KSLW toward OCLN, 96-well flat microtiter plates were coated with each construct in 20 mM carbonate–bicarbonate buffer (pH 9.6) containing 0.02% sodium azide and were incubated overnight at 4°C. Next, the plates were washed three times with TBS buffer (0.1 M NaCl and 0.02 M Tris-HCl [pH 7.4]) containing 0.05% Tween 20 and were incubated with full-length OCLN or OCLN extracellular domain B or D (50–5000 nM) diluted in the buffer for 1 h. The plates were rinsed and were incubated with rabbit anti-OCLN polyclonal antibody (dilution, 1:1000) for 1 h. Next, the plates were washed and incubated with goat anti-rabbit IgG (dilution, 1:1000; KPL, Gaithersburg, MD) for 1 h. After washing, the plates were incubated with 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonate) (KPL). Colorimetric analysis was performed by measuring absorbance at 405 nm.

Immunoprecipitation and western blotting: HUVECs were treated with KSLW, PEG5K–KSLW, or PLM–KSLW for 6 h, followed by treatment with LPS (100 ng/mL) for 12 h, and were lysed in immunoprecipitation (IP) buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 150 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitors). Cells lysates were cleared by centrifugation at 12,000 × *g* and 4°C for 10 min and were incubated overnight with anti-OCLN antibody at 4°C. Immunoprecipitates were recovered using protein A–Sepharose beads, washed four times with IP buffer, resuspended in SDS-PAGE sample buffer, and boiled for 10 min. Bound proteins were analyzed by immunoblotting with anti-phosphorylated Tyr, anti-phosphorylated Thr, anti-phosphorylated Ser, or anti-OCLN antibodies.

Gelatin zymography: Enzymatic activities of MMP-2 and MMP-9 in culture media were determined by performing SDS-PAGE gelatin zymography. Gelatinases present in the plasma degrade gelatin matrix, producing a clear band when analyzed by performing western blotting [11]. Briefly, media obtained from LPS- and PLM–KSLW-treated HUVECs (normalized to an equal amount of protein [20 μg]) were denatured in the absence of a reducing agent and were electrophoresed by performing SDS–PAGE on 10% gels containing 0.1% (w/v) gelatin. The gels were incubated with 2.5% Triton X-100 at room temperature for 2 h, followed by overnight incubation with a buffer containing 10 mM $CaCl_2$, 0.15 M NaCl, and 50 mM Tris (pH 7.5) at 37°C. Next, the gels were stained with 0.25% Coomassie Blue, and proteolysis was detected as a white band against a blue background.

Histologic analysis of OCLN cleavage in vivo: KSLW, PEG5K–KSLW, or PLM–KSLW (500 μg/kg) was intravenously injected into mice at 24 h after CLP. After 24 h, the mouse vena cava was enucleated and fixed in Visikol for 24 h. Next, the vena cava was embedded in optimum cutting temperature compound (Tissue-Tek) at -80°C. Consecutive sections (10 mm) were incubated with anti-OCLN antibody, anti-rabbit Alexa 647 (green), anti-CD31 antibody, and anti-rabbit Alexa 488 (red) and were visualized by performing confocal microscopy (TCS-SP5; Leica Microsystem, Germany) at 63× magnification.

Bacterial count: Peritoneal lavage fluids and blood were collected at 24 h after CLP. The samples were separated by centrifugation at 13,000 rpm for 10 min. Diluted samples were cultured on blood–agar base plates (trypticase soy agar plates; BD) and were incubated at 37°C for 24 h. Bacterial count was

determined by counting colony-forming units.

Determination of bactericidal activity: A direct killing effect of KSLW, PEG5K–KSLW, or PLM–KSLW was examined using gram-negative and -positive bacteria. LB plates containing the indicated concentrations of KSLW, PEG5K–KSLW, or PLM–KSLW were seeded with the specified gram-negative and -positive bacteria, and surviving colonies were counted. As a control, an LB plate containing gentamicin for gram-negative bacteria and vancomycin against gram-positive bacteria was used.

Clinical chemistry and cytokine levels in the plasma of septic mice: Fresh serum was used for assaying AST, ALT, BUN, creatinine, and LDH levels by using biochemical kits (Mybiosource). IFN-γ, IL-1β, -2, -4, -5, -6, -10, -13, TNF-α, and MCP-1 levels were determined using commercially available ELISA kits (R&D Systems), according to the manufacturer's protocol. The values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria).

Antibody measurement: KSLW or PLM–KSLW (500 μg/kg) were intravenously injected into the mice twice (immediately and after 38 h). 96-well plastic flat microtiter plates (Corning, NY, USA) were coated with fresh serum (200 μL) in 20 mM carbonate-bicarbonate buffer (pH 9.6) with 0.02% sodium azide for 1.5 h at 37°C. IgG_{2A} or IgG_{2B} antibodies (100 μL, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added to the plates and incubated for 1 h at 37°C. After washing with phosphate buffered saline (PBS)-0.05% Tween 20 (PBS-T) thrice, plates were blocked for 2 h with 200 μL of PBS containing 10% bovine serum albumin. The plates were rinsed three times with PBS-T and incubated for 1.5 h with

peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.). The plates were rinsed three times with PBS-T and incubated for 60 min at room temperature in the dark with 200 μL substrate solution (100 μg/mL *o*-phenylenediamine (OPD, Sigma) and 0.003% H_2O_2). Values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria). All measurements were performed in triplicate wells.

Expression of CAMs: Expression of VCAM-1, ICAM-1, and E-selectin on HUVECs was determined by performing whole-cell ELISA, as described previously [13, 14]. Briefly, confluent monolayers of HUVECs were treated with KSLW, PEG5K–KSLW, or PLM–KSLW for 6 h, followed by treatment with LPS (1 μg/mL) for 6 h (VCAM-1 and ICAM-1) or 10 h (E-Selectin). The medium was removed, and the cells were washed with PBS and fixed in 1% paraformaldehyde (50 μL) for 15 min at room temperature. After washing, the cells were treated with mouse anti-human VCAM-1 monoclonal antibody (100 μL; clone, 6C7.1), anti-human ICAM-1 antibody (clone, P2A4), and anti-human E-selectin antibody (clone, P2H3) (dilution, 1:50 each; Millipore Corporation). After incubation for 1 h at 37°C in 5% CO_2, the cells were washed three times and were treated with peroxidase-conjugated anti-mouse IgG (100 μL; dilution, 1:2000; Sigma, Saint Louis, MO) for 1 h. The cells were washed again three times and were developed using OPD substrate (Sigma). Colorimetric analysis was performed by measuring absorbance at 490 nm using an ELISA plate reader (Tecan, Austria GmbH). All measurements were performed in triplicate wells.

Cell–cell adhesion assay: Adherence of monocytes to endothelial cells was evaluated by fluorescently labeling the monocytes, as described previously [2, 12]. Briefly, the monocytes were labeled with 5 μM

Vybrant DiD for 20 min at 37°C in phenol red-free RPMI containing 5% fetal bovine serum (FBS). After washing, the cells (1.5×10^6 cells/mL and 200 μL/well) were resuspended in the adhesion medium (RPMI containing 2% FBS and 20 mM HEPES). The cells were then added to the confluent monolayers of HUVECs in 96-well plates. Before the addition of monocytes, HUVECs were treated with KSLW, PEG5K–KSLW, or PLM–KSLW for 6 h, followed by treatment with LPS (1 μg/mL) for 4 h. Cell adhesion was quantified, as described previously [2].

In vitro cell migration assay: Cell migration assays were performed using Transwell plates with a diameter of 6.5 mm and with filters having a pore size of 8 μm. HUVECs (6×10^4 cells/well) were cultured for three days to obtain confluent monolayers. Before adding monocytes to the upper compartment, cell monolayers were treated with LPS (1 μg/mL) for 4 h, followed by treatment with KSLW, PEG5K–KSLW, or PLM–KSLW for 6 h. Cells in the upper chamber of the filter were aspirated, and non-migrating cells on top of the filter were removed using a cotton swab. Monocytes on the lower side of the filter were fixed with 8% glutaraldehyde and were stained with 0.25% crystal violet in 20% w/v methanol. Each experiment was repeated in duplicate wells. Within each well, cells in nine randomly selected high-power microscopic fields (magnification, 200×) were counted and were expressed as migration index.

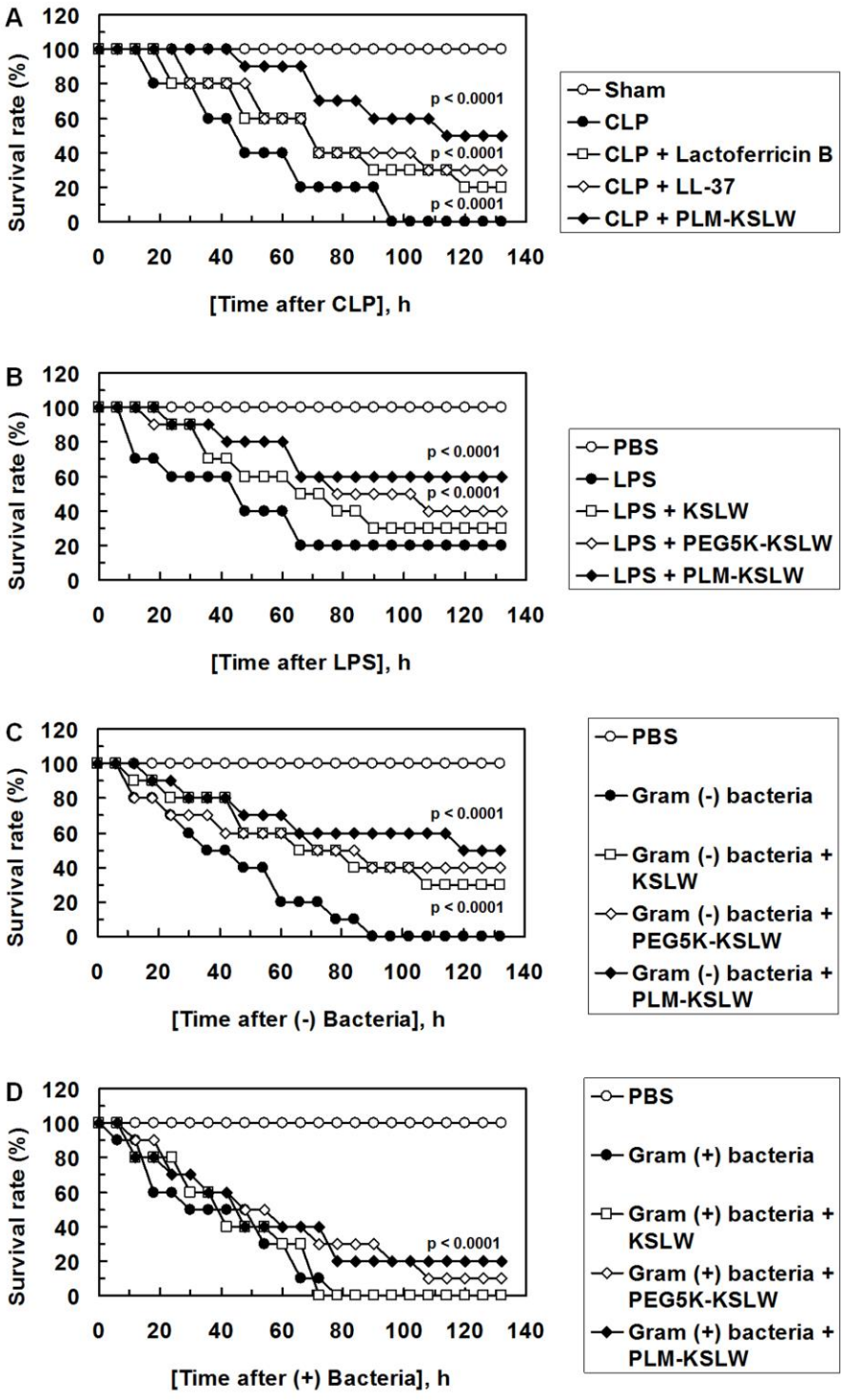
In vivo total leukocyte migration assay: For assessing total leukocyte migration, CLP-operated mice were treated with each construct (500 μg/kg) for 6 h after CLP. The mice were then sacrificed, and their peritoneal cavities were washed with 5 mL normal saline. Peritoneal fluid (20 μL) was mixed with Turk's

solution (0.38 mL; 0.01% crystal violet in 3% acetic acid), and the number of leukocytes was counted under an optical microscope. Results are expressed as neutrophils $\times 10^6$ per peritoneal cavity.

Statistical analysis: All experiments were performed independently at least three times. Values are expressed as mean ± standard deviation (SD). Statistical significance of differences between the test groups was evaluated using SPSS for Windows, version 16.0 (SPSS, Chicago, IL). Statistical relevance was determined using one-way analysis of variance and Tukey's post-hoc test. *P* values of <0.05 were considered statistically significant. Survival rates of CLP-induced septic mice were determined by performing Kaplan–Meier analysis. Data of bacterial count were analyzed using Mann–Whitney *U* test.

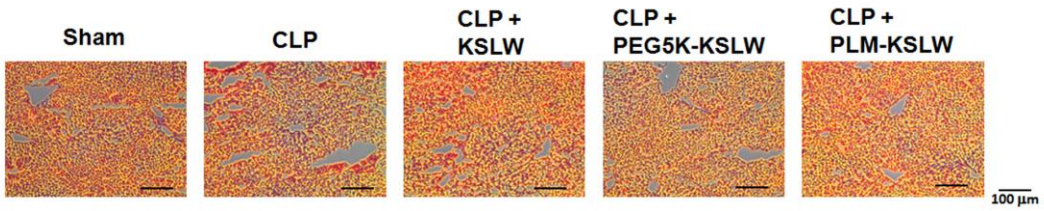
Supplementary Figure Captions

Figure S1. Effects of KSLW on the survival rate of septic mice. (A) Male C57BL/6 mice (*n* = 20) were treated with LL-37, lactoferricin B, or PLM–KSLW (500 μg/kg) at 12 and 50 h after CLP. Control CLP-operated mice (○) and sham-operated mice (●) were treated with sterile saline. Survival rate was monitored for 5 days after CLP. (B) C57BL/6 mice (n = 20) were intravenously injected with a lethal dose of LPS (30 mg/kg). Next, KSLW, PEG5K–KSLW, or PLM–KSLW (500 μg/kg) were intravenously injected into the mice at 12 and 50 h after the LPS injection. Survival rate was monitored for 5 days after LPS injection. (C and D) Protective effect of KSLW on the survival rate of mice challenged with a lethal dose of gram-negative (C) or gram-positive (D) bacteria. C57BL/6 mice were injected with 5×10^8 CFU of each bacterial species and were intravenously injected with KSLW, PEG5K–KSLW, or PLM–KSLW at 12 and 50 h after the bacterial injection.


A
Survival rate (%)
[Time after CLP], h
p < 0.0001
p < 0.0001
p < 0.0001
Sham
CLP
CLP + Lactoferricin B
CLP + LL-37
CLP + PLM-KSLW
B
Survival rate (%)
[Time after LPS], h
p < 0.0001
p < 0.0001
PBS
LPS
LPS + KSLW
LPS + PEG5K-KSLW
LPS + PLM-KSLW
C
Survival rate (%)
[Time after (-) Bacteria], h
p < 0.0001
p < 0.0001
PBS
Gram (-) bacteria
Gram (-) bacteria + KSLW
Gram (-) bacteria + PEG5K-KSLW
Gram (-) bacteria + PLM-KSLW
D
Survival rate (%)
[Time after (+) Bacteria], h
p < 0.0001
PBS
Gram (+) bacteria
Gram (+) bacteria + KSLW
Gram (+) bacteria + PEG5K-KSLW
Gram (+) bacteria + PLM-KSLW

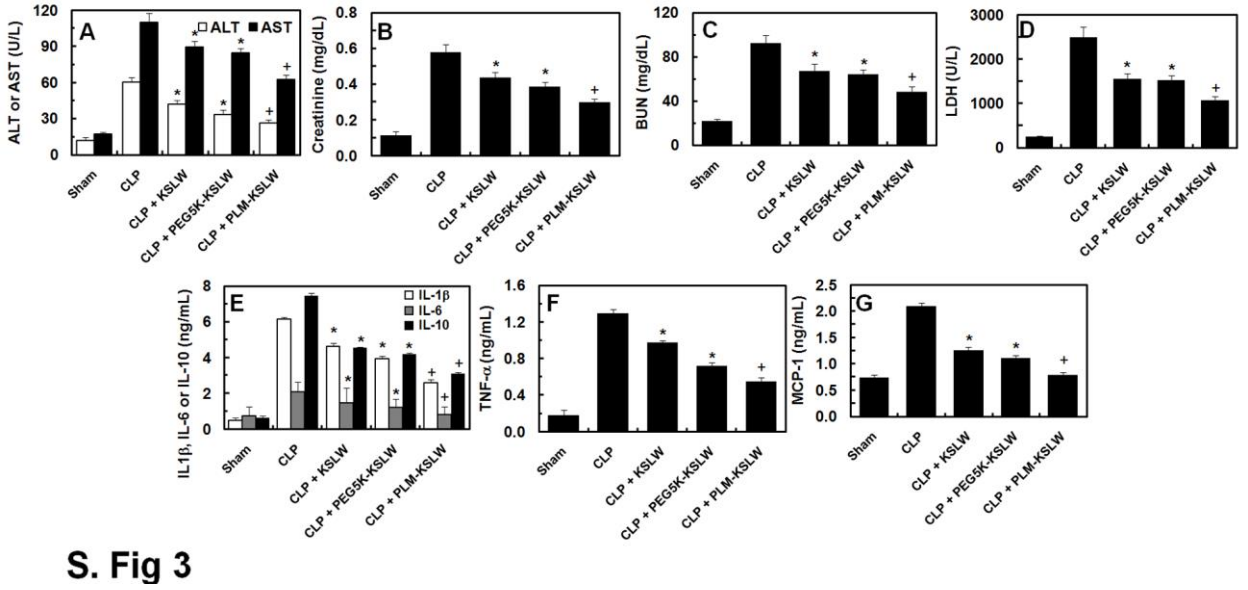
S. Fig 1

Figure S2. **Effects of PEG–KSLW and PLM–KSLW on the histological changes in liver tissues after CLP**. Male C57BL/6 mice were subjected to CLP, administered KSLW, PEG–KSLW, or PLM–KSLW (500 μg/kg) intravenously at 12 h and 50 h after CLP (n = 5), and euthanized at 96 h after CLP. Histological examination of liver sections (H&E staining, original magnification 200 x). The images are representative of three separate experiments conducted on different days with similar results.



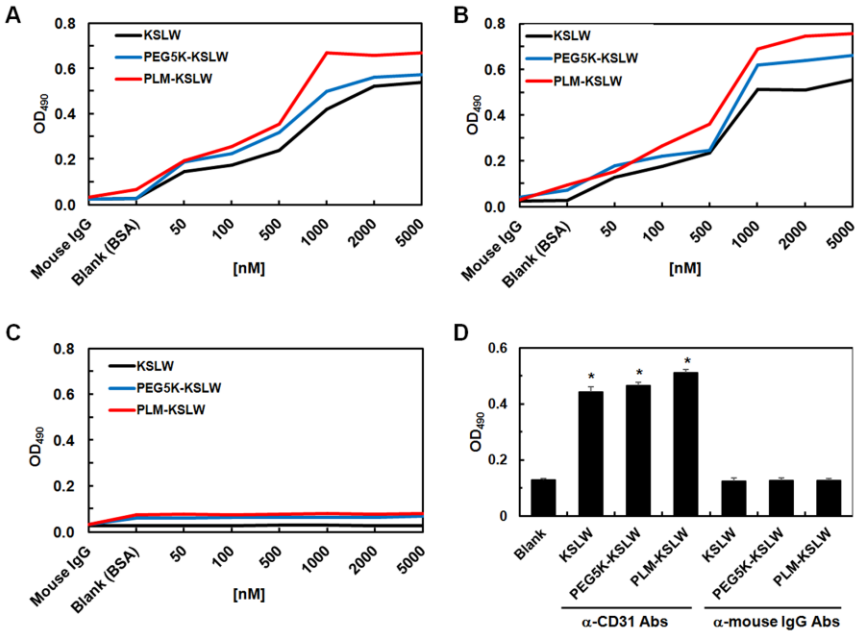
S. Fig 2

Figure S3. KSLW, PEG–KSLW, and PLM–KSLW reduce CLP-induced organ damage and cytokine production. Levels of hepatic injury markers AST and ALT (A); renal injury markers creatinine (B) and BUN (C); and tissue injury markers LDH (D), IL-1β, IL-6, IL-10 (E), TNF-α (F), and MCP-1 (G) were measured (n = 5) at 72 h after CLP; $^{*}p < 0.05$ *vs*. CLP only treatment, $^{+}p < 0.05$ *vs*. KSLW or PEG–KSLW treatment.



S. Fig 3

Figure S4. (A-C) Binding properties of KSLW, PEG5K–KSLW, and PLM–KSLW to the extracellular domains of OCLN were determined by performing ELISA. Each KSLW construct was coated on a plate and was reacted with full-length OCLN (A) and extracellular domain B (B) and extracellular domain D (C) of OCLN at concentrations up to 5000 nM for 1 h. (D) Attachment of HUVECs to KSLW, PEG5K–KSLW, or PLM–KSLW. Each construct (2 mM) were coated on non-cell adherent plates and HUVECs were incubated. After washing with PBS, CD31 ELISA method was used to detect the attachment of HUVECs to each construct as described in the Methods section. $^{*}p < 0.05$ *vs*. Blank.



S. Fig 4

Figure S5. Expression and western blotting analysis of full-length OCLN and extracellular domains B and D of OCLN. Identities of human full-length OCLN (A) and the extracellular domains of OCLN (B) were compared with those of commercial recombinant human OCLN (Abcam) by performing western blotting.

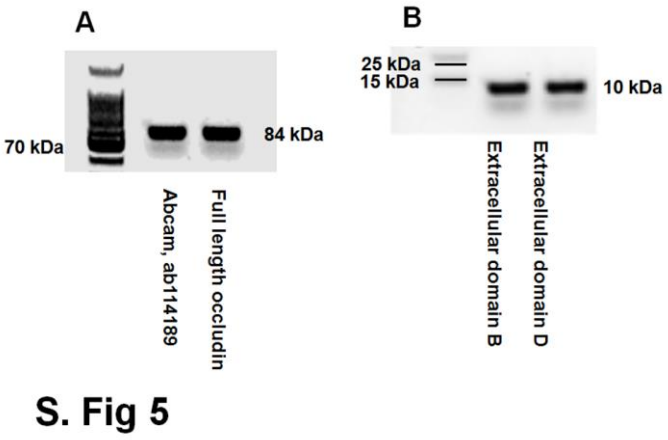
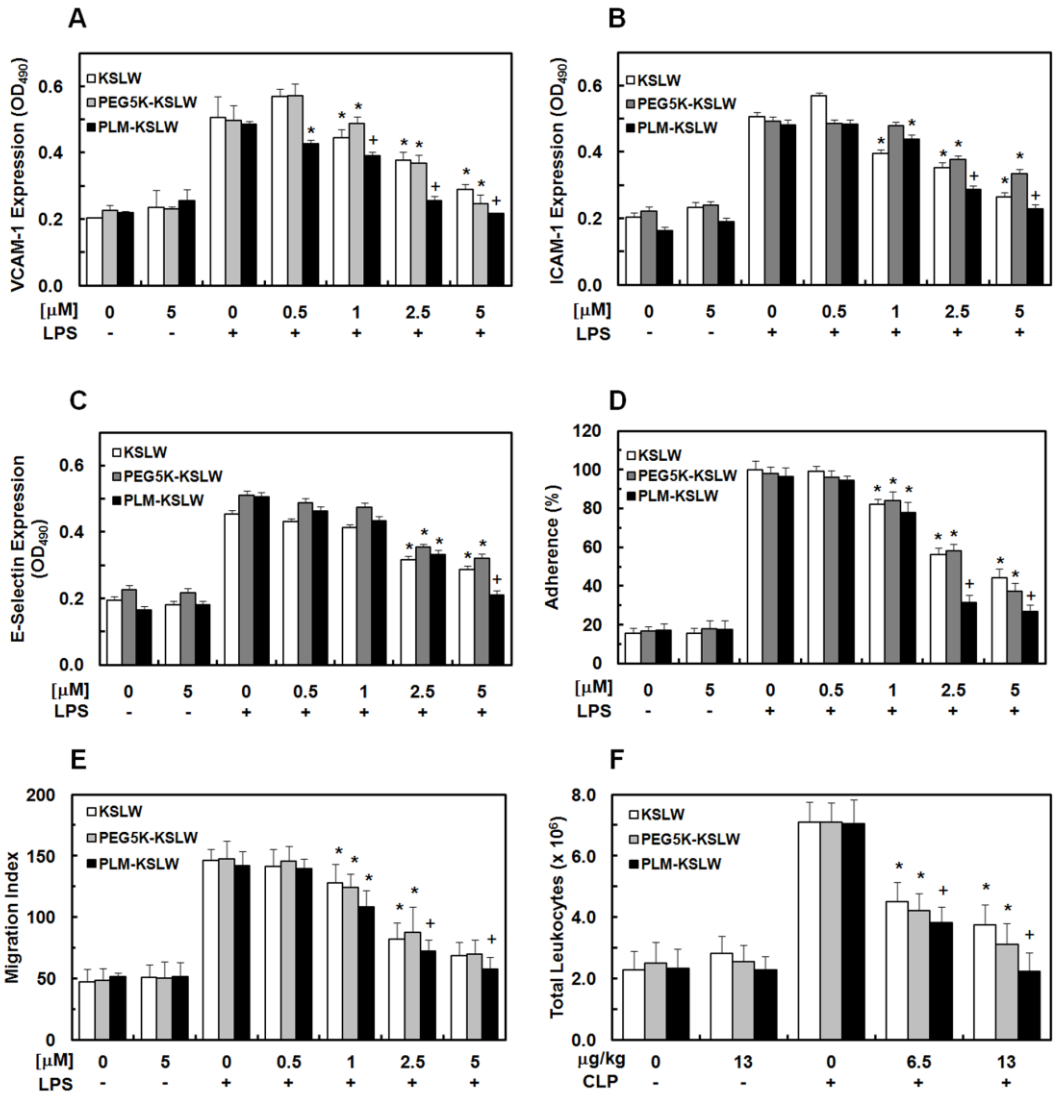


Figure S6. KSLW, PEG–KSLW, and PLM–KSLW suppressed LPS- or CLP-mediated vascular inflammatory responses. LPS (1 μg/mL)-treated HUVECs (A–E) or CLP-operated mice (6 h, F) were treated with the indicated concentrations of KSLW, PEG–KSLW, and PLM–KSLW. Expression levels of VCAM-1 (A), ICAM-1 (B), or E-selectin (C); adherence of human leukocytes to HUVEC monolayers (D); and migration of leukocytes into HUVECs (E) or peritoneal cavities of mice (F) were determined. All results are expressed as mean ± SD of three independent experiments performed on different days; $^{*}p < 0.05$ *vs*. LPS only treatment (A–E) or CLP only treatment (F).



S. Fig 6

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