Anti-Inflammatory Effects of Lysozyme Against HMGB1 in Human Endothelial Cells and in Mice

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Abstract—High mobility group box 1 (HMGB1) was recently shown to be an important extracellular mediator of severe vascular inflammatory disease, sepsis. Lysozyme protects us from the ever-present danger of bacterial infection and binds to bacterial lipopolysaccharide (LPS) with a high affinity. Here, we show, for the first time, the anti-septic effects of lysozyme in HMGB1-mediated inflammatory responses *in vitro* and *in vivo*. The data showed that lysozyme posttreatment suppressed LPS-mediated release of HMGB1 and HMGB1-mediated cytoskeletal rearrangement. Lysozyme also inhibited HMGB1-mediated hyperpermeability and leukocyte migration in human endothelial cells. In addition, lysozyme inhibited the HMGB1-mediated activation of Akt, nuclear factor-κB (NF-κB), extracellular regulated kinases (ERK) 1/2 and production of interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and chemoattractant protein-1 (MCP-1) in HUVECs. Furthermore, lysozyme reduced the cecal ligation and puncture (CLP)-induced release of HMGB1, migration of leukocytes, septic mortality, and pulmonary damage in mice. Collectively, these results suggest lysozyme as a candidate therapeutic agent for the treatment of vascular inflammatory diseases *via* inhibition of the HMGB1 signaling pathway.

KEY WORDS: lysozyme; HMGB1; vascular inflammation; sepsis.

INTRODUCTION

Sepsis is a systemic inflammatory response to presumed or known infection [1]. It is a leading cause of in-hospital death in adults, and its incidence is increasing worldwide [1]. High mobility group box1 (HMGB1) is a nonhistone chromosomal protein with high electrophoretic mobility [2]. Its proinflammatory properties, acquired upon its cellular release and consequent receptor stimulation, contribute to the pathogenesis of various human diseases [3, 4]. The recent discovery of HMGB1, as a critical

mediator of sepsis, stimulated an increasing interest in inflammation research field [3, 4]. The kinetics of HMGB1 release is delayed relative to the classical early cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). HMGB1 binds to several transmembrane receptors, such as receptor for advanced glycation end products (RAGE) and toll-like receptor (TLR)-2 and TLR-4, and activates NF-κB and extracellular regulated kinase (ERK) 1 and ERK 2 [5, 6]. Activation of endothelial cells by HMGB1 induces the expression of cell adhesion molecules (CAMs) such as vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and E-selectin, which upregulates inflammation through the recruitment of leukocytes [7]. HMGB1 accumulates during sepsis, leading to multiple organ collapse and death [8]. Therefore, HMGB1 is a therapeutic target for the clinical management of lethal systemic inflammatory diseases.

Lysozymes, found in great concentrations in blood, saliva, tears, and milk, to thwart bacterial growth, are 1,4-β-*N*-acetylmuramidases, cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid (NAM) and the C-4 of N-acetylglucosamine (NAG) in the bacterial peptidoglycan (PG) [9]. It protects us from the ever-present danger of bacterial infection [10, 11]. It is a small enzyme that

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attacks the protective cell walls of bacteria [10, 11]. Bacteria build a tough skin of carbohydrate chains, interlocked by short peptide strands, which braces their delicate membrane against the cell's high osmotic pressure [10, 11]. Lysozyme breaks these carbohydrate chains, destroying the structural integrity of the cell wall [10, 11]. The bacteria burst under their own internal pressure. Lysozyme can destroy these bacteria, and it is a catalytic enzyme responsible for the hydrolysis of the structural polysaccharides of bacterial cell walls [10, 11]. Because it is commonly found in the spots where microorganisms are most likely to enter the body, lysozyme is one of the powerful first line defenses against bacterial infection [10, 11]. Based on its biological effects, we hypothesized that lysozyme could be used to treat bacterial sepsis. To the best of our knowledge, we describe here, for the first time, the effects of lysozyme on HMGB1 release, HMGB1-mediated proinflammatory responses, and the molecular mechanisms underlying the barrier protective effects of lysozyme both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents

Lysozyme from chicken egg white (L7651), bacterial lipopolysaccharide (LPS; serotype: 0111:B4, L5293), Evans blue dye, crystal violet, and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan). Fetal bovine serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bioscience (Charles City, IA) and cultured in endothelial cell growth medium (EGM; Cambrex Bioscience) containing 10 % fetal bovine serum (FBS, HyClone Laboratories, Logan, UT). HUVECs were used in cell culture at passages 3–5 as described previously [12–16]. Human neutrophils were freshly isolated from whole blood (15 mL) obtained by venipuncture from five healthy volunteers and maintained as previously described [17, 18].

Animals and Husbandry

Six to 7-week-old C57BL/6 male mice weighing 25–28 g were purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea) and were used in this study after a 12-day acclimatization period. Animals were housed five per polycarbonate cage under controlled temperature (20–25 °C) and humidity (40–45 %) and a 12:12-h light/dark cycle. During acclimatization, animals were supplied a normal rodent pellet diet and water *ad libitum*. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Kyungpook National University, Republic of Korea (IRB approval number, KNU 2012-13).

Cecal Ligation and Puncture (CLP)

For induction of sepsis, 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* a facemask. They were allowed to breathe spontaneously during the procedure. The CLP-induced sepsis model was prepared as previously described [13, 19]. In brief, a 2-cm midline incision was made to expose the cecum and adjoining intestine. The cecum then was tightly ligated with a 3.0-silk suture 5.0 mm from the cecal tip and was punctured once using a 22-gauge needle for induction of high-grade sepsis [20]. Then, the cecum was gently squeezed to extrude a small amount of feces from the perforation site and was returned to the peritoneal cavity. Finally, the laparotomy site was sutured with 4.0-silk. In sham control animals, the cecum was exposed but not ligated or punctured and then was returned to the abdominal cavity. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2012-13).

Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the HMGB1 concentration in cell culture media or in mice serum, competitive ELISA was performed as previously described [21]. HUVEC monolayers were treated with LPS (100 ng/mL) for 16 h, followed by treatment with lysozyme from 0 to 200 nM for 6 h. Then, cell culture media was collected for the determination of HMGB1 concentration. For ELISA, 96-well flat plastic microtiter plates (Corning Inc., Corning, NY) were coated overnight at 4 °C with HMGB1 protein in 20 mM carbonate-bicarbonate buffer (pH 9.6) containing 0.02 % sodium azide. Plates then were rinsed three times in PBS-0.05 % Tween 20 (PBS-T) and were kept at 4 °C. Lyophilized culture media was preincubated with anti-HMGB1 antibodies (diluted 1:1000 in PBS-T, Abnova, Taipei City, Taiwan) in 96-well round plastic microtiter plates for

90 min at 37 °C, transferred to precoated plates, and incubated for 30 min at room temperature. Plates were then rinsed three times in PBS-T, incubated for 90 min at room temperature with peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2000 in PBS-T, GE Healthcare Life Science, Pittsburgh), rinsed three times with PBS-T, and incubated for 60 min at room temperature in the dark with 200 µL of substrate solution (100 µg/mL o-phenylenediamine and 0.003 % H_2O_2). Reactions were stopped with 50 µL of 8N H_2SO_4.

To determine the concentrations of CAM such as VCAM-1, ICAM-1, and E-selectin, whole cell ELISA was applied as previously described [22, 23]. Briefly, confluent monolayers of HUVECs were treated with HMGB1 (1 µg/mL) for 16 h (VCAM-1 and ICAM-1) or 22 h (E-selectin), followed by treatment with lysozyme from 0 to 200 nM, and were fixed in 1 % paraformaldehyde. After washing three times, mouse anti-human monoclonal antibodies (VCAM-1, ICAM-1, and E-selectin, diluted 1:50 in PBS-T, Temecula, CA) were added, followed by incubation of the cells for 1 h (37 °C, 5 % CO_2). Cells then were washed, treated with peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 1 h, and washed three times, followed by development using o-phenylenediamine substrate (Sigma). The same experimental procedures were used for monitoring the cell surface expression of the HMGB1 receptors, TLR2, TLR4, and RAGE, using specific antibodies (A-9, H-80, and A-9, respectively) obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). To determine the concentrations of VCAM-1, ICAM-1, and E-selectin in mouse endothelial cells derived from CLP-induced, commercially available ELISA kits were used according to the manufacturer's instructions (Abcam, Cambridge, MA).

To determine the concentrations of phosphorylated p38 MAPK, nuclear factor (NF)-κB, MCP-1, Akt, TNF-α, ERK 1/2, IL-1β, and IL-6 commercially available ELISA kits were used according to the manufacturer's supplied protocol: MCP-1, IL-1β, L-6, total and phosphorylated ERK 1/2, TNF-α (R&D Systems, Minneapolis, MN), total and phosphorylated Akt, phosphorylated p38, total and phosphorylated p65 NF-κB (#7170, #7252 Cell Signaling Technology, Danvers, MA). Values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria).

Cell Viability Assay

The MTT assay was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium, followed by treatment with lysozyme from 0 to 200 nM. After a 48-h incubation period, cells were washed, and 100 µL of MTT (1 mg/mL) was added, followed by incubation for 4 h. Finally, dimethyl sulfoxide (DMSO) (150 µL) was added to solubilize the formazan salt formed, and the amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (Tecan Austria GmbH, Austria).

RNA Isolation and Real-Time PCR

RNA was isolated by using TRI-Reagent (Invitrogen) according to the manufacturer's suggested protocol. An aliquot (5 µg) of extract RNA was reverse transcribed into first-strand cDNA with a PX2 Thermal Cycler (Thermo Scientific) using 200 U/µL M-MLV reverse-transcriptase (Invitrogen) and 0.5 mg/µL of oligo(dT)-adapter primer (Invitrogen) in a 20-µL reaction mixture. Real-time PCR for VCAM-1, ICAM-1, E-selectin, and α-actin was carried out with a Mini Opticon Real-Time PCR System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The primers had the following sequences: for VCAM-1, sense 5′-TGG AGG AAA TGG GCA TAA AG-3′ and antisense 5′-CAG GAT TTT GGG AGC TGG TA-3′; for ICAM-1, sense 5′-CGA AGG TTC TTC TGA GC-3′ and antisense 5′-GTC TGC TGA GAC CCC TCT TG-3′; for E-selectin, sense 5′-TCT GGA CCT TTC CAA AAT GG-3′ and antisense 5′-TGC AAG CTA AAG CCC TCA TT-3′; and for α-actin, sense 5′-TGA GAG GGA AAT CGT GCG TG-3′ and antisense 5′-TTG CTG ATC CAC ATC TGC TGG-3′; for TLR2, sense 5′-ATC CTC CAA TCA GGC TTC TCT-3′ and antisence 5′- ACA CCT CTG TAG GTC ACT GTT G-3′; for HMGB1, sense 5′-GGA CAA GGC CCG TTA TGA AAG AGA AAT GA-3′ and antisense 5′- AGC AGA AGA GGA AGA AGG CCG AAG GAG-3′. The PCR settings were as follows: initial denaturation at 95 °C was followed by 35 cycles of amplification for 15 s at 95 °C and 20 s at 60 °C, with subsequent melting curve analysis, increasing the temperature from 72 to 98 °C. Quantification of gene expression was calculated relative to α-actin.

Permeability Assay In Vitro

Endothelial cell permeability after exposure to lysozyme from 0 to 200 nM was quantified by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers using a modified two-compartment chamber model, as previously described [24]. HUVECs were plated (5×10^4/well) in 3-µm pore

size, 12-mm diameter transwells and incubated for 3 days. Confluent monolayers of HUVECs were treated with HMGB1 (1 μg/mL) for 16 h, and then treated with lysozyme for 6 h.

In Vitro Migration Assay

Migration assays were performed in 6.5-mm diameter transwell plates containing 8-μm pore size filters. HUVECs (6×10^4) were cultured for 3 days to obtain confluent endothelial monolayers. Before the addition of human neutrophils to the upper compartment, cell monolayers were treated with HMGB1 (1 μg/mL) for 16 h, and then treated with lysozyme for 6 h. Transwell plates were then incubated at 37 °C in 5 % CO_2 for 2 h. Cells in the upper chamber were then aspirated, and non-migrating cells on top of the filter were removed using a cotton swab. Human neutrophils on the lower side of the filter were fixed with 8 % glutaraldehyde and stained with 0.25 % crystal violet in 20 % methanol (w/v). Each experiment was repeated twice in duplicate wells, and nine randomly selected high power microscopic fields (HPF; 200×) were counted. The results are expressed as migration indices.

In Vivo Permeability and Leukocyte Migration Assays

For the in vivo study, male mice were anesthetized with zoletil (tiletamine and zolazepam 1:1 mixture, 30 mg/kg) and rompun (xylazine, 10 mg/kg). CLP-operated mice or mice pretreated with HMGB1 (2 μg/ mouse, i.v.) for 16 h were injected with lysozyme (0.57, 1.43, 2.86, or 5.72 μg per mouse i.v.). After 6 h, 1 % Evans blue dye solution in normal saline was injected i.v. into each mouse. Thirty minutes later, the mice were sacrificed, and the peritoneal exudates were collected after washing with 5 mL of normal saline and centrifuged at 200×g for 10 min. The absorbance of the supernatant was measured at 650 nm. Vascular permeability was expressed in terms of the amount of dye (μg per mouse) that leaked into the peritoneal cavity according to a standard curve of Evans blue dye, as previously described [25].

To assess leukocyte migration, CLP-operated mice and mice treated with HMGB1 (2 μg per mouse, i.v.) in normal saline for 16 h were treated with lysozyme (0.57, 1.43, 2.86, or 5.72 μg per mouse i.v.) for 6 h. The mice were then sacrificed, and the peritoneal cavities were washed with 5 mL of normal saline. Twenty microliters of peritoneal fluid was mixed with 0.38 mL of Turk's solution (0.01 % crystal violet in 3 % acetic acid), and the number of leukocytes was counted under a light microscope.

Cell-Cell Adhesion Assay

Human neutrophil adherence to HUVECs was evaluated by fluorescent labeling of neutrophils as previously described [26]. Briefly, neutrophils (1.5×10^6/mL, 200 μL/ well) were labeled with Vybrant DiD dye, and then added to washed and stimulated HUVECs. HUVEC monolayers were treated with HMGB1 (1 μg/mL) for 16 h, and then treated with lysozyme for 6 h.

Hematoxylin and Eosin Staining and Histopathological Examination

Male C57BL/6 mice underwent CLP and were administered lysozyme (2.86 or 5.72 μg per mouse i.v.) at 12 and 50 h after CLP ($n=5$). Mice were euthanized 96 h after CLP. To analyze the phenotypic change of lung tissue in mouse, lung samples were removed from each mouse, washed three times in PBS (pH 7.4) to remove remaining 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 through an ethanol series, embedded in paraffin, sectioned (4-μm thickness), and placed on a slide. The slides were deparaffinized in a 60 °C oven, rehydrated, and stained with hematoxylin (Sigma). To remove excess stain, the slides were quick-dipped three times in 0.3 % acid alcohol and were counterstained with eosin (Sigma). Excess stain then was removed in an ethanol series and xylene, before slides were mounted with coverslips. Light microscopic analysis of lung specimens was performed by blinded observation to evaluate pulmonary architecture, tissue edema, and infiltration of inflammatory cells as previously defined [27]. The results 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 partial destruction of pulmonary architecture; and grade 4 included dense neutrophil leukocyte infiltration, abscess formation, and complete destruction of pulmonary architecture.

Immunofluorescence Staining

HUVECs were grown to confluence on glass cover slips coated with 0.05 % poly-L-lysine in complete media containing 10 % FBS and maintained for 48 h. Cells were then stimulated with HMGB1 (1 μg/mL) for 16 h with or without 6-h lysozyme treatment (50 or 100 nM). For cytoskeletal staining, the cells were fixed in 4 % formaldehyde in PBS (v/v) for 15 min at room temperature,

permeabilized in 0.05 % Triton X-100 in PBS for 15 min, and blocked in blocking buffer (5 % BSA in PBS) overnight at 4 °C. Then, the cells were incubated with F-actin-labeled fluorescein phalloidin (F 432; Molecular Probes, Invitrogen) or primary rabbit monoclonal NF-κB p65 antibody and anti-rabbit alexa 488 overnight at 4 °C. Nuclei were counterstained with 4,6-diamidino-2-phenylindole dihydroChloride (DAPI) and were visualized by confocal microscopy at a ×630 magnification (TCS-Sp5, Leica microsystem, Germany).

Statistical Analysis

Each experiment was independently repeated at least three times. All data are indicated as mean±standard error of the mean (SEM). The statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS, Chicago, IL). Significant differences between groups were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc test. P values less than 0.05 were considered statistically significant. A survival analysis of CLP-induced sepsis was performed using the Kaplan–Meier method.

RESULTS AND DISCUSSION

Effect of Lysozyme on LPS- and CLP-Mediated Release of HMGB1

Previous studies have shown that LPS stimulates the release of HMGB1 from murine macrophages and human endothelial cells [7, 28]. In agreement with these previous results, LPS (100 ng/mL) stimulated the release of HMGB1 from HUVECs (Fig. 1a). To investigate the effects of lysozyme on LPS-mediated release of HMGB1, endothelial cells were stimulated with 100 ng/mL LPS for 16 h before treatment with increasing concentrations of lysozyme for 6 h. The results shown in Fig. 1a indicate that lysozyme inhibits the LPS-induced release of HMGB1 from endothelial cells, and the optimal effect was observed at concentrations greater than 20 nM. However, lysozyme treatment alone did not affect HMGB1 release (Fig. 1a). To confirm this effect *in vivo*, we used a standardized mouse model of sepsis, in which severe sepsis was induced by cecal ligation and puncture (CLP) because this model closely resembles human sepsis [29]. As shown in Fig. 1b, lysozyme treatment marked inhibited CLP-induced release of HMGB1 in mice. The average circulating blood volume for mice is 72 mL/kg [30]. Because the average weight of used mouse is 27 g, and the average

blood volume is 2 mL, the amount of lysozyme (0.57, 1.43, 2.86, or 5.72 μg per mouse) injected yielded a maximum concentration of 20, 50, 100, or 200 nM in the peripheral blood. Maximum amounts of used lysozyme (5.72 μg per mouse=212 μg/kg) administered into mouse is safe because previous reports used up to 100 mg/kg [31], 300 mg/kg [32], or 1000 mg/kg [33]. To determine the molecular mechanism by which lysozyme inhibited the release of LPS-mediated HMGB1, we tested the effects of lysozyme on the transcriptional regulation of HMGB1 by LPS in HUVECs. Thus, we measured the effect of lysozyme on LPS-induced HMGB1 mRNA levels using real-time qRT-PCR. As shown in Fig. 1c, LPS induced an increase in the expression levels of HMGB1 mRNA and treatment with lysozyme resulted in decreased expression levels of LPS-induced HMGB1 mRNA. Next, we investigated the effects of lysozyme on the expression of the HMGB1 receptors TLR2, TLR4, and RAGE in HUVECs. As shown in Fig. 1d, HMGB1 treatment induced a 4-fold increase in the expression of TLR-2, TLR-4, and RAGE in HUVECs. Lysozyme treatment markedly inhibited TLR2, TLR4, and RAGE expression. To test the effects of lysozyme on cellular viability, MTT assays were performed in HUVECs treated with lysozyme for 24 h. Lysozyme did not affect cell viability at the concentrations used (up to 200 nM) (Fig. 1e). High plasma concentrations of HMGB1 in patients with inflammatory diseases are known to be related to poor prognosis and high mortality. In addition, pharmacological inhibition of HMGB1 is known to improve survival in animal models of acute inflammation in response to endotoxin challenge [34]. Therefore, the prevention of LPS- or CLP-induced release of HMGB1 by lysozyme suggests that lysozyme could be used for the treatment of vascular inflammatory diseases.

Effect of Lysozyme on LPS-, HMGB1-, or CLP-Mediated Barrier Disruption

A permeability assay was performed to determine the effects of lysozyme on the barrier integrity of HUVECs. Treatment with lysozyme (200 nM) alone did not alter barrier integrity (Fig. 2a). In contrast, LPS is known to induce the cleavage and disruption of endothelial membrane barriers [35, 36]. HUVECs were treated with various concentrations of lysozyme for 6 h after the addition of LPS (100 ng/mL). As shown in Fig. 2a, lysozyme down-regulated LPS-mediated membrane disruption in a dose-dependent manner. HMGB1 is also known to induce cleavage and disruption of barrier integrity [37, 38]. Treatment with lysozyme resulted in a dose-dependent decrease in
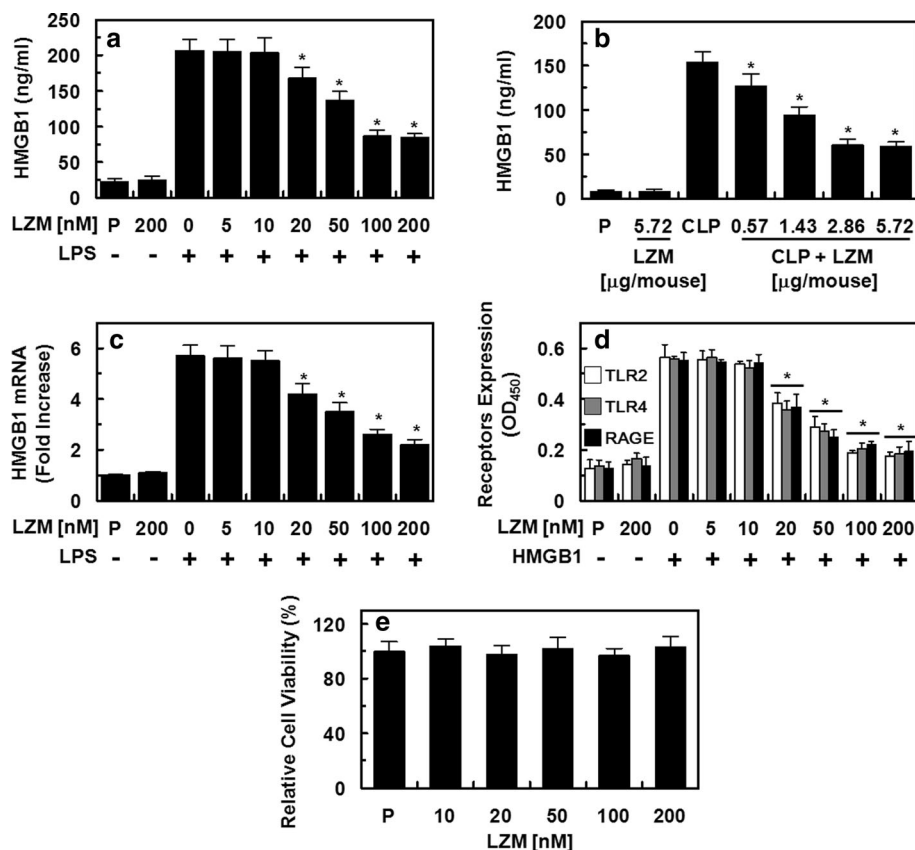
Fig. 1. The effects of lysozyme on HMGB1 release and the expression of HMGB1 receptors. **a** HUVECs were treated with the indicated concentrations of lysozyme for 6 h after stimulation with LPS (100 ng/mL) for 16 h. HMGB1 release was then measured by ELISA. **b** Male C57BL/6 mice underwent CLP and were then administered lysozyme (0.57–5.72 μg per mouse) intravenously (i.v.) 12 h after CLP ($n=5$). Mice were euthanized 24 h after CLP. Serum HMGB1 levels were measured by ELISA. **c** The same as **a** except that real-time qRT-PCR analysis was performed using specific primers for HMGB1 and actin, as described in the "MATERIALS AND METHODS" section. **d** Confluent HUVECs were incubated with HMGB1 (1 μg/mL) for 16 h. The cells were then treated with the indicated concentrations of lysozyme for 6 h. Expression of the HMGB1 receptors TLR-2, TLR-4, and RAGE in HUVECs was measured by cell-based ELISA. **e** Effect of lysozyme on cell viability was measured by MTT assay. Results are expressed as the mean±SEM of five independent experiments. *$p<0.05$ versus LPS alone (**a**, **c**), CLP (**b**), or HMGB1 alone (**d**). *P* PBS vehicle control.

HMGB1-mediated membrane disruption (Fig. 2b). To confirm this effect *in vivo*, HMGB1- or CLP-induced vascular permeability in mice was evaluated. As shown in Fig. 2c, lysozyme markedly inhibited HMGB1- or CLP-induced peritoneal leakage of dye. HMGB1 is known to induce proinflammatory responses by promoting phosphorylation of p38 MAPK [39, 40]. To determine whether lysozyme inhibits HMGB1-induced activation of p38 MAPK in HUVECs, cells were activated with HMGB1 and incubated with lysozyme, and then phosphorylated p38 MAPK levels were determined. As shown in Fig. 2d, HMGB1 upregulated the expression of phosphorylated p38, which was significantly inhibited by lysozyme treatment. These findings demonstrate that lysozyme treatment inhibited HMGB1-mediated endothelial disruption and maintained human endothelial cell barrier integrity in mice.

Cytoskeletal proteins are important for the maintenance of cell integrity and shape [41]. In addition, redistribution of the actin cytoskeleton, detachment of cells, and loss of cell-cell contact due to cytokine stimulation are all associated with an increased endothelial monolayer permeability [42, 43]. Therefore, we next examined the effects of lysozyme on actin cytoskeletal arrangement in HUVECs by immunofluorescence staining of HUVEC monolayers with F-actin-labeled fluorescein phalloidin. Control HUVECs exhibited a random distribution of F-actin throughout the cells, with some localization of actin filament bundles at the cell boundaries (Fig. 2e). Barrier disruption in HUVECs induced by HMGB1 treatment (1 μg/mL) was accompanied by the formation of paracellular gaps (shown by arrows). A similar cytoskeletal arrangement was induced by LPS treatment (100 ng/mL)
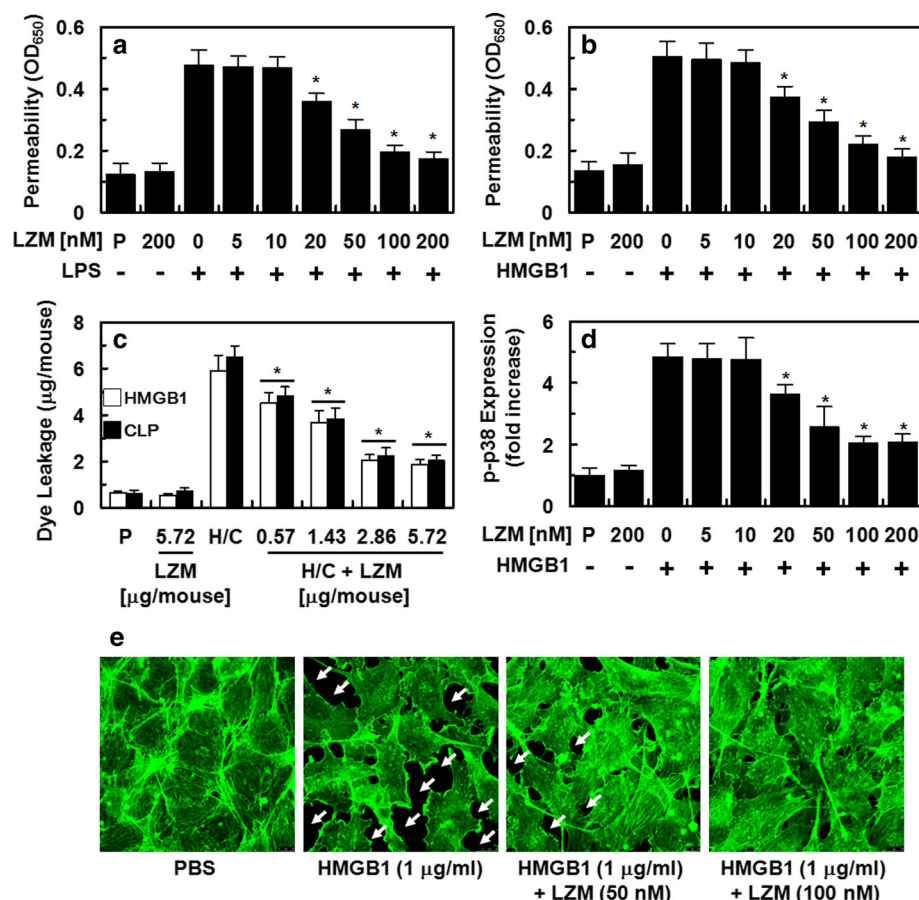
Fig. 2. Effects of lysozyme on HMGB1-mediated permeability *in vitro* and *in vivo*. **a–b** HUVECs were stimulated with LPS (**a**, 100 ng/mL for 4 h) or HMGB1 (**b**, 1 μg/mL for 16 h), and then treated with different concentrations of lysozyme for 6 h. Permeability was then monitored by measuring the flux of Evans blue dye-bound albumin across HUVECs. **c** The effects of lysozyme (0.57–5.72 μg per mouse) injected in HMGB1- (H, 2 μg/mouse, i.v.) or CLP- (**c**, at 24 h after CLP) induced vascular permeability in mice were examined by measuring the amount of Evans blue dye in peritoneal washes (expressed in μg per mouse, $n = 5$). **d** HUVECs were activated with HMGB1 and were then treated with different concentrations of lysozyme for 6 h. The effects of lysozyme on HMGB1-mediated expression of phospho-p38 were measured by ELISA. **e** Staining for F-actin. HUVEC monolayers grown on glass coverslips were stimulated with HMGB1 for 1 h, and then treated with lysozyme (50 or 100 nM) for 6 h, and stained for F-actin. *Arrows* indicate intercellular gaps. Results are expressed as the mean±SEM of five independent experiments. *$p < 0.05$ versus LPS (**a**), HMGB1 (**b**, **d**), or CLP/HMGB1 (**d**). *P* PBS vehicle control.

(data not shown). In addition, treatment with lysozyme (50 or 100 nM) inhibited the formation of HMGB1-induced paracellular gaps with the formation of dense F-actin rings (Fig. 2e). These results suggest that lysozyme treatment inhibited the HMGB1-mediated morphological changes and gap formation in endothelial cells, which are associated with F-actin redistribution, thereby increasing vascular barrier integrity.

Lysozyme Inhibited the Expression of CAMs and Proinflammatory Responses

Previous studies have demonstrated that HMGB1 mediates inflammatory responses by increasing the cell surface expression of CAMs, such as ICAM-1, VCAM-1, and E-selectin, on the surface of endothelial cells, thereby promoting the adhesion and migration of leukocytes across the endothelium to the sites of inflammation [44]. To determine the effects of lysozyme on the expression of CAMs in HMGB1-stimulated endothelial cells, we monitored the expression of VCAM-1, ICAM-1, and E-selectin in HMGB1-stimulated, lysozyme-treated HUVECs. According to our findings, HMGB1 induced upregulation of the surface protein expression of VCAM-1, ICAM-1, and E-selectin (Fig. 3a), and transcription levels of each (Fig. 3b) and lysozyme inhibited this effect, suggesting that the inhibitory effects of lysozyme on expression of CAMs are mediated *via* attenuation of the HMGB1 signaling pathway
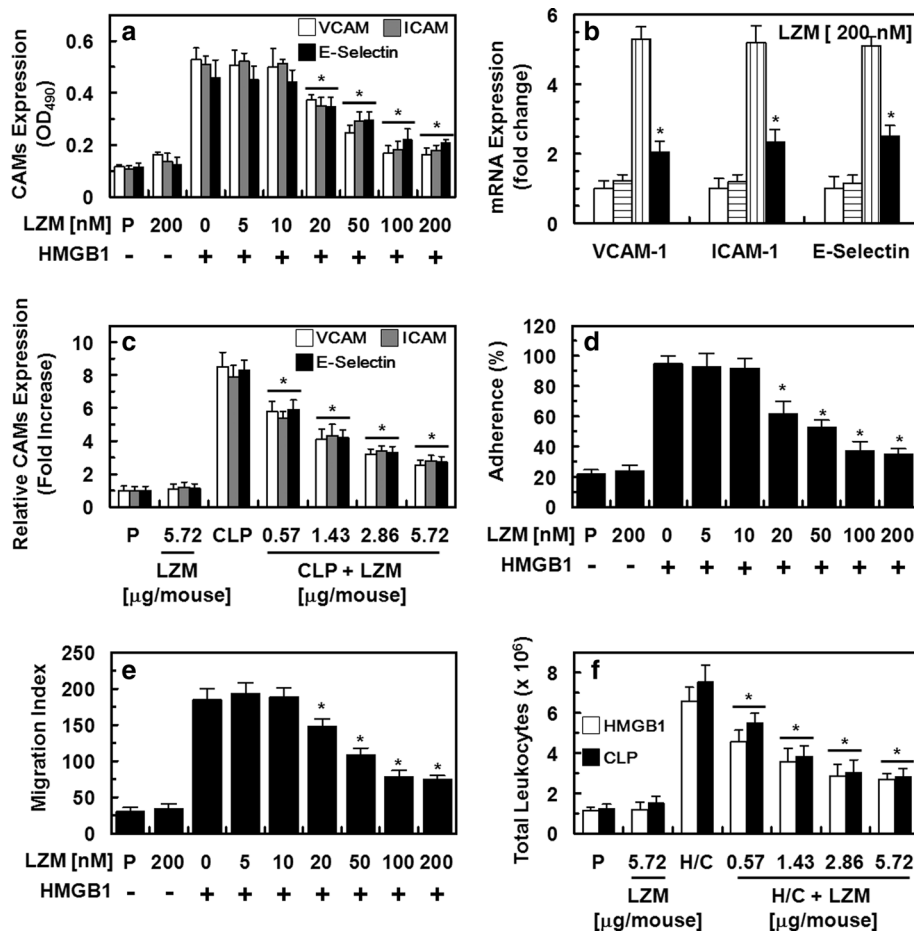
Fig. 3. Effect of lysozyme on HMGB1-mediated proinflammatory responses. HUVECs were stimulated with HMGB1 (1 μg/mL) for 16 h, and then treated with various concentrations of lysozyme for 6 h. **a** HMGB1-mediated expression of VCAM-1, ICAM-1, and E-selectin in HUVECs, **d** adherence of human neutrophils to HUVEC monolayers, and **e** migration of human neutrophils through HUVEC monolayers were analyzed. **b** HMGB1-mediated (1 μg/mL) transcription level (mRNA) of VCAM-1, ICAM-1, and E-selectin in HUVECs was analyzed after treating monolayers with 200 nM lysozyme by Q-PCR. Control (□), lysozyme alone ( ), HMGB1 alone ( ), and HMGB1 + lysozyme (■). **c, f** CLP-operated (at 24 h after CLP), and then injected with lysozyme (0.57–5.72 μg per mouse, i.v.). **f** C57BL/6 male mice were stimulated with HMGB1 (H, 2 μg per mouse, i.v.) and then injected with lysozyme (0.57–5.72 μg per mouse, i.v.). CLP-induced expressions of CAMs (**c**) or HMGB1- or CLP-mediated migration of leukocytes into the peritoneal cavity of mice (**f**) were analyzed. Results are expressed as the mean±SEM of five independent experiments. *$p < 0.05$ versus HMGB1 (**a, b, d–f**) or CLP (**c, f**). P PBS vehicle control.

by lysozyme. We confirmed these results in CLP-induced *in vivo* conditions (Fig. 3c). The adhesion of leukocytes to endothelial cells and the transendothelial migration (TEM) of leukocytes are important steps in the proinflammatory response [45]. Therefore, we attempted to determine whether the expression of CAMs corresponded to enhanced leukocyte binding and whether lysozyme could block the adhesion of monocytes to HMGB1-stimulated HUVECs. We found that lysozyme effectively inhibited the binding of human neutrophils to HMGB1-stimulated HUVECs (Fig. 3d). Further studies revealed a relationship between the binding of neutrophils to HUVECs and the subsequent TEM of neutrophils and showed that lysozyme effectively

inhibited this step (Fig. 3e). To confirm this effect *in vivo*, HMGB1- or CLP-induced leukocyte migration in the presence of lysozyme was examined in mice. HMGB1 and CLP significantly stimulated the migration of leukocytes into the peritoneal cavities of mice. Lysozyme at doses of 20–200 nM significantly inhibited this migration (Fig. 3f). These results indicate that lysozyme not only inhibits the endotoxin-mediated release of HMGB1 from endothelial cells but also downregulates the proinflammatory signaling effect of released HMGB1, thereby inhibiting the amplification of steps in the inflammatory pathway, such as the upregulation of CAMs and leukocyte adhesion and migration induced by HMGB1.

Lysozyme Inhibited HMGB1-Stimulated Activation of NF-κB, ERK, and Akt as Well as the Oroduction of TNF-α, IL-6, IL-1β, and MCP-1

Both HMGB1 and LPS induced a significant increase in the nuclear translocation of NF-κB and the phosphorylation of Akt and p38 MAPK in human endothelial cells [46, 47]. Activation of NF-κB and ERK1/2 is required for proinflammatory responses [48, 49]. Previous studies have reported the activation of NF-κB and ERK 1/2 by HMGB1 in vascular inflammatory responses [50, 51]. Therefore, to investigate the potential effects of lysozyme on the activation of inflammatory signaling molecules in HMGB1-activated HUVECs, the cells were activated by treatment with HMGB1 for 16 h, and then incubated with lysozyme for 6 h. The data showed thephosphorylation of NF-κB (Fig. 4a), ERK1/2 (Fig. 4b), and Akt (Fig. 4c). And, we measured HMGB1-induced translocation of NF-κB from cytosol to nucleus by using p65 NF-κB and fluorescein isothiocyanate (FITC)-conjugated antibody. As a result, immunofluorescence staining (Fig. 4d) showed that stimulation with HMGB1 resulted in obvious translocation of NF-κB p65 from cytoplasm into nucleus, which was obviously counteracted by treatment with lysozyme. To support these inhibitory effects of lysozyme, the effects of lysozyme on the production of TNF-α and IL-1β in HMGB1-activated HUVECs were tested. To do this, the cells were activated by treatment with HMGB1 for 16 h, and then incubated with lysozyme for 6 h. The data showed that the production of TNF-α (Fig. 4e) and IL-1β (Fig. 4g) were increased by treatment with HMGB1, and that these increases were significantly reduced by lysozyme treatment at concentrations >20 nM. Lysozyme also inhibited other inflammatory markers such as IL-6 and MCP-1 as shown in Fig. 4i, k. The inhibitory effects of lysozyme on the productions of TNF-α, IL-β, and IL-6 were also confirmed in CLP-induced *in vivo* conditions (Fig. 4f, h, j). These results indicate that lysozyme may affect the most important signals involved in the induction of proinflammatory responses in human endothelial cells.

The Protective Effect of Lysozyme in CLP-Induced Septic Mortality

Sepsis, a systemic response to a serious infection, has a poor prognosis when it is associated with organ dysfunction, hypoperfusion, or hypotension [4, 52]. Based on the above-mentioned findings, we hypothesized that treatment with lysozyme would reduce mortality in our CLP-induced sepsis mouse model. To determine whether lysozyme protects mice from CLP-induced sepsis lethality, mice were administered lysozyme after CLP surgery. Twenty-four hours after the CLP operation, the animals manifested signs of sepsis, including shivering, bristled hair, and weakness. Administration of lysozyme once at two different doses (2.86 or 5.72 μg per mouse, 12 h after CLP) did not prevent CLP-induced death (data not shown). Thus, in a subsequent experiment, lysozyme was administered twice, once 12 h after CLP and a second time 50 h after CLP, and Kaplan-Meier survival analysis revealed a significant increase in this treatment group ($p < 0.0001$, Fig. 5a). The final survival rates 132 h after CLP was 20 % for the 2.86 μg per mouse group and 40 % for the 5.72 μg per mouse group. These marked benefits of lysozyme administration, including suppression of HMGB1 release and HMGB1-mediated inflammatory responses, offer a therapeutic strategy for the management of sepsis and septic shock.

Protective Effect of Lysozyme in the CLP-Induced Pulmonary Injury

To confirm the protective effects of lysozyme on CLP-induced death, we determined the effects of lysozyme on CLP-induced pulmonary injury. There were no significant differences between lungs of sham and sham + lysozyme in light microscopic observations (data not shown). In the CLP group, interstitial edema with massive infiltration of the inflammatory cells into the interstitium and alveolar spaces were observed, and the pulmonary architecture was severely damaged (Fig. 5b, c). These morphological changes were less pronounced, and the pulmonary architecture was preserved, and lung injury score was reduced in the CLP + lysozyme groups than in the CLP group (Fig. 5b, c).

Sepsis is a systemic inflammatory response syndrome, resulting from microbial infection. A wide array of proinflammatory cytokines, including TNF-α, IL-1β, interferon-γ, and macrophage migration inhibitory factor, either individually or in combination, contribute to the pathogenesis of lethal systemic inflammation [53]. For instance, neutralizing antibodies against TNF-α [54] reduce lethality in an animal model of endotoxemic/bacteremic shock. However, the early kinetics of systemic TNF-α accumulation makes its targeting difficult in the clinical setting [54], which prompted the investigation of other late proinflammatory mediators such as HMGB1 as potential therapeutic targets for inflammatory diseases. The prevailing theories of sepsis as a deregulated systemic inflammatory response are supported by extensive studies
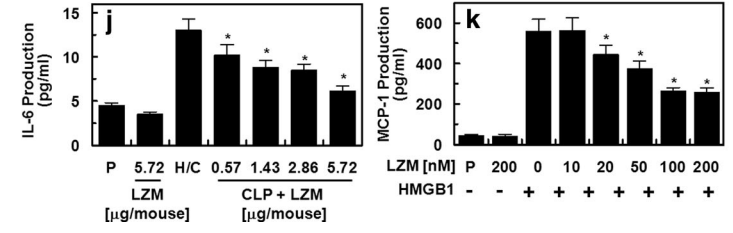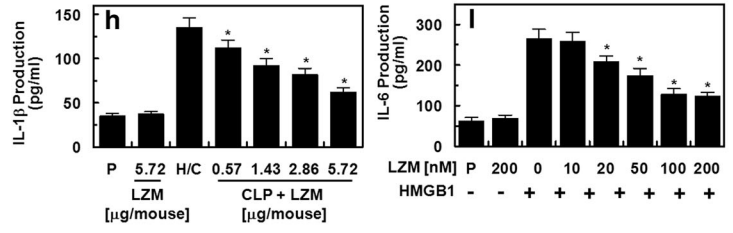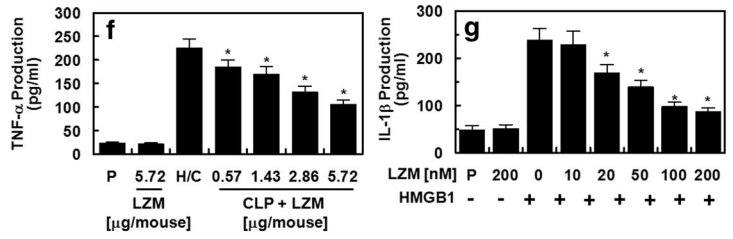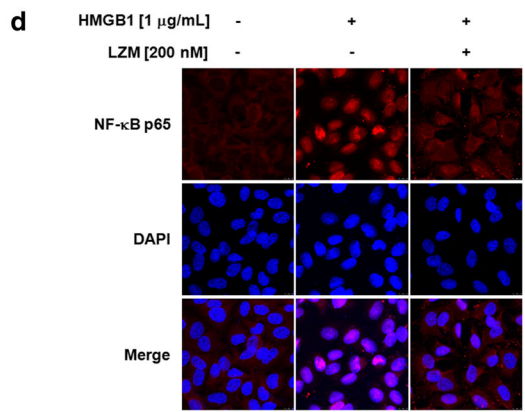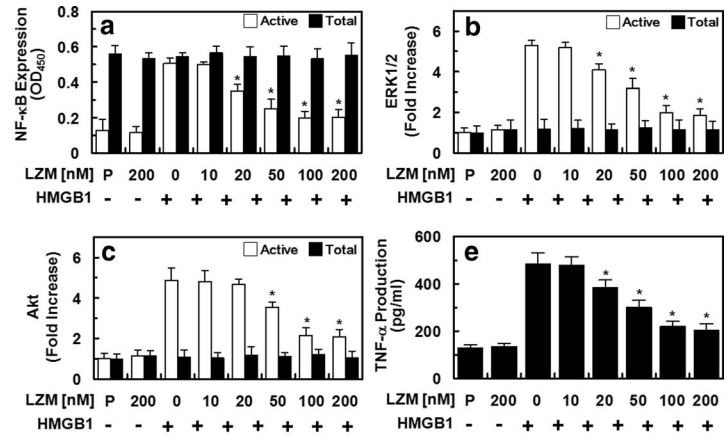
Fig. 4. Effects of lysozyme on HMGB1-stimulated activation of NF-κB, ERK, and Akt and the production of TNF-α, IL-1β, IL-6, and MCP-1 in HUVECs. HUVECs were stimulated with HMGB1 (1 μg/mL) for 16 h and then treated with various concentrations of lysozyme for 6 h. **a**–**c** HMGB1-mediated (1 μg/mL) activation of NF-κB p65 (**a**), ERK1/2 (**b**), and Akt (**c**) levels in HUVECs were analyzed. HMGB1 (1 μg/mL)-mediated production of TNF-α (**e**), IL-1β (**g**), IL-6 (**i**), and MCP-1 (**k**) in HUVECs was analyzed. **d** Immunofluorescence microscopy analysis of the nuclear translocation of p65 in HUVECs. HUVECs were stimulated (or not) for 1 h with 1 μg/mL HMGB1 and treated or not with 200 nM lysozyme for 6 h. The subcellular localization of p65 was examined by IF staining. **f**, **h**, **j** CLP-operated, and then injected with lysozyme (0.57–5.72 μg per mouse, i.v.). The productions of TNF-α (at 8 h after CLP, **e**), IL-1β (at 24 h after CLP, **g**), and IL-6 (at 24 h after CLP, **i**) were analyzed. Results are expressed as the mean±SEM of five independent experiments. *$p < 0.05$ versus HMGB1. *P* PBS vehicle control.

employing various animal models of sepsis, including endotoxemia and peritonitis induced by CLP [55]. In murine models of endotoxemia and sepsis, HMGB1 is first detectable in the circulating blood 8 h after the onset of the disease, and subsequently reaches a plateau at 16 to 32 h after onset [56]. This late appearance of circulating HMGB1 precedes and parallels with the onset of animal lethality from endotoxemia or sepsis and distinguishes it from TNF-α and other early proinflammatory cytokines [56]. Therefore, in this study, lysozyme was administered 16 h after the inflammatory challenge.

Endotoxic LPS is well known as an important contributing factor to the pathogenesis of Gram-negative bacterial infections and has the capacity to interact with a variety of host target cells and molecules [57]. And, it induces in both humans and experimental animals septic shock which is characterized by hypotension, disseminated intravascular coagulation, and multiple organ failure [57]. Previous reports showed that lysozyme inhibited several immunostimulatory activities of natural LPS by forming a complex *in vitro* [58–60]. Noting that lysozyme could bind to LPS with high affinity [59, 60], the inhibitory effects of
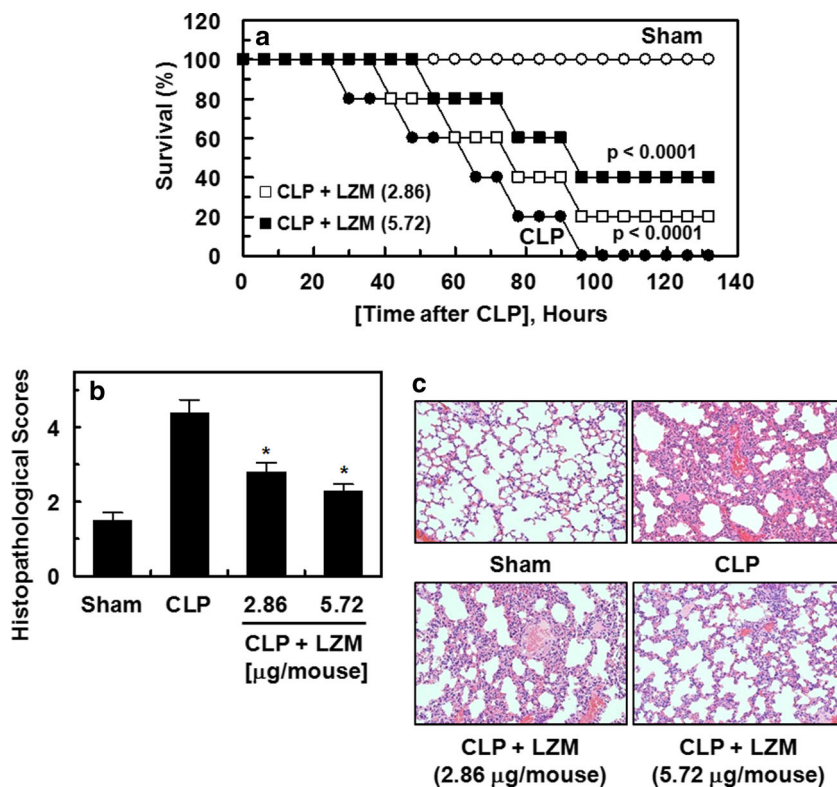

Fig. 5. Effects of lysozyme on lethality or pulmonary injury after CLP. **a** Male C57BL/6 mice ($n=20$) were administered lysozyme (2.86 or 5.72 μg per mouse) intravenously at 12 and 50 h after CLP. Animal survival was monitored every 6 h after CLP for 132 h. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline. Kaplan-Meier survival analysis was used for determination of overall survival rates versus CLP-treated mice. **b** Male C57BL/6 mice underwent CLP and were administered lysozyme (2.86 or 5.72 μg per mouse) intravenously at 12 and 50 h after CLP ($n=5$). Mice were euthanized 96 h after CLP. Histopathological scores of the lung tissue were recorded as described in methods. *$p < 0.05$ vs HMGB1 alone. **c** Photomicrographs of lung tissues (H&E staining, ×200). *Images* are representative of three independent experiments.

lysozyme on the LPS-mediated HMGB1 secretion may be not due to the binding of lysozyme to LPS because in this study, lysozyme was administrated after LPS treatment.

Based on the original data and revised data, therefore, the anti-inflammatory effects of lysozyme against HMGB1-mediated septic response may be mediated by the suppression of HMGB1 release (Fig. 1a, b), the expressions of HMGB1 receptors such as TLR2, TLR4, and RAGE (Fig. 1d) and HMGB1-mediated hyperpermeability (Fig. 2b, c, e) *via* suppression of the activation of p38 (Fig. 2d). Furthermore, the inhibitory mechanism of lysozyme on the interaction of between leukocytes and endothelial cells is mediated by the inhibition of the expressions of CAMs such as VCAM, ICAM, and E-selectin (Fig. 3). The underlying mechanism of these anti-inflammatory effects of lysozyme is the suppression of LPS-mediated HMGB1 mRNA expressions (Fig. 1c), the downregulation of the production of TNF-α, IL-1β, and -6, and the activation of NF-κB, Akt, and ERK1/2 (Fig. 4). Lysozyme also inhibited the translocation of NF-κb from cytosol to nucleus (Fig. 4d).

Recently, the only FDA-approved drug for severe sepsis, recombinant human activated protein C (marketed as Xigris), was withdrawn from the market due to lack of beneficial effects on 28-day mortality in the PROWESS and septic shock (PROWESS-SHOCK) trials [61]. As a result, we are now left with no effective preventative or treatment option for severe sepsis. Thus, based on the current study, lysozyme treatment could be another strategy for developing drug candidates against sepsis. In summary, our results demonstrate that lysozyme inhibits both LPS- and CLP-mediated release of HMGB1, the expression of the HMGB1 receptors TLR2, 4, and RAGE, and HMGB1-mediated barrier disruption by increasing barrier integrity and inhibiting CAMs expression. These protective effects of lysozyme were confirmed in a mouse model of sepsis, in which lysozyme treatment reduced HMGB1-induced mortality. Our findings indicate that lysozyme may be a candidate for use in the treatment of severe vascular inflammatory diseases such as septic shock.

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