

Transient Receptor Potential Melastatin 2 Protects Mice against Polymicrobial Sepsis by Enhancing Bacterial Clearance

XiaoWei Qian, M.Sc., Tomohiro Numata, Ph.D., Kai Zhang, M.Sc., CaiXia Li, M.Sc., JinChao Hou, M.Sc., Yasuo Mori, Ph.D., XiangMing Fang, M.D.

ABSTRACT

Background: Recent studies suggest that the transient receptor potential melastatin 2 (TRPM2) channel plays an important role in inflammation and immune response. However, the role and mechanism of TRPM2 in polymicrobial sepsis remain unclear.

Methods: The authors explored the effects of genetic disruption of TRPM2 on mortality ($n = 15$), bacterial clearance ($n = 6$), organ injury, and systemic inflammation during cecal ligation and puncture-induced sepsis. Electrophysiology, immunoblot, bacterial clearance experiment, and quantitative real-time polymerase chain reaction were used to explore the role and mechanism of TRPM2 in sepsis.

Results: After cecal ligation and puncture, Trpm2-knockout mice had increased mortality compared with wild-type mice (73.3 vs. 40%, $P = 0.0289$). The increased mortality was associated with increased bacterial burden, organ injury, and systemic inflammation. TRPM2-mediated Ca^{2+} influx plays an important role in lipopolysaccharide or cecal ligation and puncture-induced heme oxygenase-1 (HO-1) expression in macrophage. HO-1 up-regulation decreased bacterial burden both in wild-type bone marrow-derived macrophages and in cecal ligation and puncture-induced septic wild-type mice. Disruption of TRPM2 decreased HO-1 expression and increased bacterial burden in bone marrow-derived macrophages. Pretreatment of Trpm2-knockout bone marrow-derived macrophages with HO-1 inducer markedly increased HO-1 expression and decreased bacterial burden. Pretreatment of Trpm2-knockout mice with HO-1 inducer reversed the susceptibility of Trpm2-knockout mice to sepsis by enhancing the bacterial clearance. In addition, septic patients with lower monocytic TRPM2 and HO-1 messenger RNA levels had a worse outcome compared with septic patients with normal monocytic TRPM2 and HO-1 messenger RNA levels. TRPM2 levels correlated with HO-1 levels in septic patients ($r = 0.675$, $P = 0.001$).

Conclusion: The study data demonstrate a protective role of TRPM2 in controlling bacterial clearance during polymicrobial sepsis possibly by regulating HO-1 expression. (ANESTHESIOLOGY 2014; 121:336-51)

SEPSIS is characterized as a harmful and dysregulated inflammatory response to infection.¹ Approximately 700,000 cases and 210,000 deaths occur annually in North America, resulting in an economic burden of approximately \$16.7 billion per year.² The incidence of sepsis increases at a rate of 8.7% per year, and the number of deaths continues to rise despite the many advances that have been made in critical care medicine.³ The failure of treatment for sepsis with high-dose corticosteroid,⁴ interleukin (IL)-1 receptor antagonists,^{5,6} tumor necrosis factor antagonists,^{7,8} and toll-like receptor antagonists⁹ in clinical trials led researchers to state that other key pathophysiological mechanisms of sepsis other than uncontrolled inflammation may exist. Increasing evidence supports that immunosuppression exists in septic patients and plays a central role in sepsis.¹⁰ The immune cells of both innate and adaptive systems are severely suppressed in septic patients and produce small quantities of inflammatory cytokines which are critical for host to eradicate invading pathogens.^{10,11} Macrophages serve as the first line of host

What We Already Know about This Topic

- The transient receptor potential melastatin 2 channel plays an important role in inflammation.

What This Article Tells Us That Is New

- Mice with transient receptor potential melastatin 2 genetically eliminated who were exposed to polymicrobial sepsis had increased mortality with increased bacterial burden, organ injury, and systemic inflammation. Similarly, patients who had lower monocytic transient receptor potential melastatin 2 levels had worse outcomes compared with patients with normal monocytic transient receptor potential melastatin 2 levels.

defense for killing invading microorganisms. However, in septic patients, macrophage function is severely impaired, which may promote uncontrolled microbial growth.¹²

Transient receptor potential melastatin 2 (TRPM2), a nonselective Ca^{2+} -permeable channel, is expressed abundantly in macrophages.¹³ Accumulated studies suggest an important role of TRPM2 in macrophage function.

Submitted for publication October 15, 2013. Accepted for publication April 1, 2014. From the Department of Anaesthesia, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China (X.Q., K.Z., C.L., J.H., X.F.); and Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan (T.N., Y.M.).

Copyright © 2014, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2014; 121:336-51

Disruption of TRPM2 attenuates inflammatory mediator production^{14–16} and phagocytic activity.¹⁴ TRPM2 is also necessary for the production of interferon- γ and protects mice against *Listeria monocytogenes* infection.¹⁷ In contrast, one recent study reported that TRPM2 deficiency promoted endotoxin-induced lung inflammation and injury.¹⁸ These findings suggest that TRPM2 may involve in the pathogenesis of sepsis by immunoregulating the macrophage function. However, to date, the role and the underlying mechanism of TRPM2 in polymicrobial sepsis remain insufficiently understood.

Heme oxygenase-1 (HO-1), a stress-responsive enzyme, plays a key role in protecting the host against injury during inflammation.¹⁹ Furthermore, HO-1 also plays a protective role in host defense against microbial sepsis by enhancing bacterial clearance.²⁰ A recent study indicates that calcium influx is critical for HO-1 induction.²¹ Whether TRPM2-mediated calcium influx plays a role in sepsis by regulating HO-1 expression is unknown.

In this study, we sought to investigate the role and mechanism of TRPM2 in the pathogenesis of polymicrobial sepsis. We found that mice deficient in TRPM2 had significantly increased mortality compared with the mortality of wild-type (WT) mice. The increased mortality was associated with increased bacterial burden. We uncovered an important protective role of TRPM2 in controlling bacterial clearance during polymicrobial sepsis possibly *via* regulation of HO-1 expression. Furthermore, TRPM2 levels correlated with HO-1 levels in monocytes from septic patients and contributed to the outcome of sepsis.

Materials and Methods

Mice and Sepsis Model

Trpm2-knockout (KO) mice (backcrossed for 12 generations onto the C57BL/6 background) were maintained in our laboratory. Male C57BL/6 WT mice were purchased from Zhejiang Province Experimental Animal Center. All animal experiments were approved by the Institutional Animal Care and Use Committees of Zhejiang University (Hangzhou, Zhejiang Province, People's Republic of China) and the Graduate School of Engineering, Kyoto University (Kyoto, Japan). The cecal ligation and puncture (CLP)-induced sepsis model was generated as previously described.²² After the mice were anesthetized using an intraperitoneal injection of 80 mg/kg pentobarbital, the cecum was exteriorized *via* a 1-cm abdominal midline incision and ligated using a 4-0 silk ligature at mid-way between distal pole and the base of cecum. The cecum was then punctured once through both surfaces using a 21-gauge needle at the middle of the ligation and the tip of the cecum. The cecum was replaced after extruding a small amount of fecal material, and the abdomen was then closed. All mice received 1 ml of normal saline subcutaneously after surgery. Sham CLP mice underwent the same procedure as described above but without being ligated

and punctured. On the basis of one previous report,²³ additional mice were injected with 10 mg/kg hemin (Sigma-Aldrich, St. Louis, MO) or vehicle (intraperitoneal) every other day (three times) before CLP. Hemin was dissolved in 10% ammonium hydroxide containing 0.15 M NaCl and further diluted 1:100 using 0.15 M NaCl. Hemin solution was filter-sterilized. Survival rate was monitored once daily for 7 days. Mice were randomly assigned to experimental groups. All further experiments were blinded to murine genotype and treatment.

Bacterial Burden Determination

The bacterial count was analyzed as previously described.²⁴ The blood was collected aseptically at 24 h after CLP or sham CLP surgery and then serially diluted in sterile phosphate-buffered saline (PBS). This diluent (100 μ l) was plated onto tryptic soy agar plates and incubated at 37°C. Colony-forming units (CFUs) were counted at 24 h, and results were expressed as CFU per milliliter blood. Peritoneal lavage fluid (PLF) was collected by washing the peritoneal cavity with 5-ml sterile PBS. After serial dilutions, 100 μ l of this diluent was plated on tryptic soy agar plates and incubated at 37°C. CFUs were counted at 24 h, and results were expressed as CFU per milliliter PLF. For bacterial analyses of organs, the lung, liver, and spleen were homogenized in 1 ml sterile PBS. After serial dilutions, 100 μ l of each organ sample was plated on tryptic soy agar plates and incubated at 37°C. CFUs were counted at 24 h, and results were expressed as CFU per organ.

Tissue Histological Analyses

Histological analyses were performed as described previously.^{25,26} At 24 h after CLP, the mice were anesthetized and euthanized. The left lung and left lobe of the liver were fixed in 4% paraformaldehyde for 24 h and then sectioned serially. A 4-point scale (0 denoted normal lungs; 1, mild, less than 25% lung involvement; 2, moderate, 25 to 50% lung involvement; 3, severe, 50 to 75% lung involvement; and 4, very severe, >75% lung involvement) was used to evaluate lung damage based on alveolar congestion, capillary congestion, leukocyte or neutrophil infiltration, and thickness of the alveolar wall.²⁵ Using an image analyzing system (Automated image analysis software; Olympus, Hamburg, Germany), leukocyte infiltration in the lung was estimated by quantitative morphometric analysis. Liver damage was based on necrosis characterized by loss of architecture, vacuolization, karyolysis, and increased eosinophilia. A scale of 0 to 4 (0 denoted normal liver; 1, mild; 2, moderate; 3, severe; and 4, total necrotic destruction of the liver) was used to evaluate liver damage.²⁶

Lung Wet/Dry Weight Ratio

Both lungs were removed, blotted, and weighted immediately at 24 h after CLP or sham CLP surgery. The lungs were dried at 60°C for 48 h and reweighed. A percentage

of wet-to-dry weight was used to estimate the lung wet/dry weight ratio.

Bronchoalveolar Lavage Fluid

At 24 h after CLP or sham CLP surgery, the mice were anesthetized with an intraperitoneal injection of 80 mg/kg pentobarbital. The lungs were lavaged using three separate 0.5-ml ice-cold PBS. The lavage was centrifuged at 1,500 rpm for 10 min at 4°C. Total protein levels in the supernatant were measured using a bicinchoninic acid protein assay kit (Thermo Scientific, Inc., Rockford, IL).

Serum Alanine Aminotransferase Activity Assay

Blood was collected at 24 h after CLP or sham CLP surgery. Using an alanine aminotransferase assay kit (Abcam, Cambridge, MA), serum alanine aminotransferase concentration was measured according to the manufacturer's instructions.

Cytokine Measurement

Blood was collected at 24 h after CLP or sham CLP surgery. The serum levels of IL-6 (R&D Systems, Minneapolis, MN) and high mobility group protein B1 (Shino-Test Co., Kyoto, Japan) were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Mouse Primary Bone Marrow Cell Culture

Bone marrow progenitors collected from both femur and tibia of WT or *Trpm2*-KO C57BL/6 mice were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific). After centrifuging at 2,000 rpm for 5 min, hypotonic solution was used to lyse erythrocytes. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Moregate Bio-Tech, Bulimba, Queensland, Australia), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco Invitrogen, Carlsbad, CA) in 75-cm² flasks overnight to remove matured cells. Nonadherent cells were collected, and 5×10^6 cells were differentiated in six-well plates for 4 days in RPMI 1640 medium containing 10% FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate (all from Gibco Invitrogen), and 20 ng/ml mouse granulocyte macrophage-colony stimulating factor (PeproTech, Rocky Hill, NJ). Cells were cultured in a humidified atmosphere at 37°C in 5% CO₂ and 95% air.

Electrophysiology

Whole cell patch recordings for current clamp and voltage clamp were recorded using nystatin-perforated patch technique on bone marrow-derived macrophages (BMDMs) from WT mice or *Trpm2*-KO mice at room temperature (22° to 25°C) with Axopatch 200B (Molecular Devices, Sunnyvale, CA) patch clamp amplifier as previously described.²⁷ For whole cell recordings, the Na⁺-based bath solution contained 145 mM NaCl, 0.4 mM CaCl₂, 1.2 mM MgCl₂,

11.5 mM HEPES, and 10 mM D-glucose (pH adjusted to 7.4 with NaOH, and osmolality adjusted to 320 mosmol/kg H₂O with D-mannitol). The pipette solution contained 55 mM K₂SO₄, 20 mM KCl, 5 mM MgCl₂, 0.2 mM EGTA, and 5 mM HEPES (pH adjusted to 7.4 with KOH, and osmolality adjusted to 300 mosmol/kg H₂O with D-mannitol). Ramp pulses were applied every 10 s from -100 mV to +100 mV from a holding potential of 0 mV at a speed of 4 mV/ms.

Western Blot Assay

Western blot was performed as described in one previous report.²⁸ Before boiling the lysates at 70°C for 10 min, ×4 lithium dodecyl sulfate sample buffer (Novex, Carlsbad, CA) and ×10 sample reducing agent (Novex) were added at a final concentration of ×1. Equal amounts of protein (30 µg) were added into each wells of a 12% Bis-Tris polyacrylamide gel (Novex) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were then blocked in Tris-buffered saline with 0.05% Tween-20 (Sigma-Aldrich) containing 5% nonfat dry milk for 1 h. For whole cell lysates, the membranes were incubated in primary antibody solution of HO-1 (1:1,000 dilution, monoclonal anti-HO-1 antibody; Epitomics, Inc., Burlingame, CA) and α-tubulin (as an internal standard, 1:1,000 dilution, monoclonal anti-α-tubulin antibody; Sigma-Aldrich) overnight on a shaker on ice. Three washes with Tris-buffered saline with 0.05% Tween-20 were followed by incubation of the membrane with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) solution for 1 h on a shaker at room temperature. After three washes with Tris-buffered saline with 0.05% Tween-20, the bands were detected by enhanced chemiluminescence solution (Thermo Scientific) and Kodak film (Carestream Health, Rochester, NY).

Measurement of Intracellular Ca²⁺

On the basis of a previous report,²⁹ intracellular Ca²⁺ concentration after lipopolysaccharide (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich) stimulation was measured using a VARIOSKAN Flash (Thermo Scientific), and 1×10^4 BMDMs were cultured in a 96-well plate for 24 h. After the lipopolysaccharide stimulation, cell monolayers were washed twice in PBS. Fluo-3 acetoxymethyl (2.5 µM; Dojindo Laboratories, Kumamoto, Japan) in phenol red-free 1640 RPMI medium was added to each well of the 96-well plate, and the plate was incubated at 37°C for 30 min and then washed twice in PBS to remove free fluo-3 acetoxymethyl. The intracellular Ca²⁺ fluorescence signal was measured at intervals of 15 s. The fluorescence of fluo-3 acetoxymethyl was excited at 508 nm and measured using a 527-nm filter. The changes in fluorescence intensity were denoted as lipopolysaccharide-induced changes in intracellular Ca²⁺ concentrations.

In Vitro Phagocytosis and Bacterial Killing by BMDMs

Bone marrow–derived macrophages were resuspended in RPMI 1640 medium containing 10% FBS (without antibiotics) and were plated at 2×10^5 cells per well in 24-well flat-bottom plate. Phagocytosis and bacterial killing assays were performed as described previously.³⁰ To determine the phagocytic function of macrophages, 2×10^7 *E. coli* (DH5 α ; Sigma-Aldrich) were added in the well containing 2×10^5 adherent BMDMs. Centrifuged the 24-well plate at 1,500 rpm for 2 min and incubated the plate at 37°C for 1 h. The cells were then washed using PBS and lysed with 500 μ l 0.1% Triton X-100 for 5 min. Cell lysates were serially diluted with PBS and plated on Luria-Bertani agar plates to determine phagocytic capacity by counting the number of CFU after incubating the plates at 37°C. To determine the bacterial killing capability of macrophages, 2×10^7 *E. coli* was added in the well containing 2×10^5 adherent BMDMs. After centrifuging the 24-well plate at 1,500 rpm for 2 min, incubated the plate at 37°C for 1 h. The cells were then washed with PBS three times and further cultured in medium containing gentamicin (100 μ g/ml) for 12 h at 37°C to kill extracellular bacteria so that only intracellular bacteria were quantified. After washing with PBS, the cells were lysed with 500 μ l 0.1% Triton X-100 for 5 min. Cell lysates were serially diluted with PBS and plated on Luria-Bertani agar plates to determine the bacterial killing capacity by counting the number of CFU after incubating the plates at 37°C. In some experiments, hemin was dissolved in 10% ammonium hydroxide containing 0.15 M NaCl and further diluted 1:100 using 0.15 M NaCl. Hemin and vehicle solution were filter-sterilized. After treatment with hemin or vehicle for 6 h, the supernatant was removed and the wells were washed with PBS three times and then added lipopolysaccharide or *E. coli* for further experiments.

Peritoneal Macrophage Isolation

At 24 h after CLP or sham CLP surgery, the mice were euthanized and dampened with 70% ethanol. After exposing the caudal half of abdominal wall by retraction, a 25-gauge needle was inserted into the peritoneal cavity. The needle was fixed with a vascular clamp and three separate 3 ml PBS were injected into the cavity. Slowly withdrew the lavage fluid containing peritoneal cells after gently shaking the entire body for 10 s. Lavage fluid was centrifuged and washed twice with PBS. The cell pellets were resuspended in 2 ml RPMI 1640 medium (Thermo Scientific) containing 10% FBS (Moregate BioTech), 100 units/ml penicillin, and 100 μ g/ml streptomycin and were cultured in six-well plates for 2 h in a humidified atmosphere at 37°C in 5% CO₂ and 95% air to allow macrophages to adhere. The supernatant was removed and the adherent macrophages were washed twice with PBS. The adherent macrophages were then harvested. The attached culture consisted of approximately 80% macrophages as assessed by F4/80 expression by flow cytometry assay.

Patients

This prospective study was approved by the ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, and informed consent was received for all patients from legally authorized representatives. From October 18, 2012 to March 1, 2013, 25 patients were admitted to the intensive care unit of the First Affiliated Hospital, College of Medicine, Zhejiang University and were considered for inclusion in this study if they met the criteria for severe sepsis or septic shock as defined as following: severe sepsis was defined by the presence of a known or suspected (without being microbiologically confirmed) source of infection and a systemic inflammatory response syndrome complicated by organ dysfunction.³¹ Septic shock was defined by the presence of known or suspected (without being microbiologically confirmed) source of infection, a systemic inflammatory response syndrome, and a state of persistent hypotension (a systolic arterial pressure of <90 mmHg, mean arterial pressure of <60 mmHg, or a reduction in systolic arterial pressure of >40 mmHg from baseline despite adequate volume resuscitation) in the absence of other causes of hypotension.³¹ Exclusion criteria for the current study included the following: age younger than 18 yr, with human immunodeficiency virus infection, treatment with corticosteroids or chemotherapy within 4 weeks, or inability to provide informed consent. Ten patients in the intensive care unit who did not show features of systemic inflammatory response syndrome or any evidence of infection were enrolled as nonseptic control patients. Clinical and demographic data were recorded within the first 24 h after diagnosis of severe sepsis or septic shock by two senior intensivists. Acute Physiologic and Chronic Health Evaluation II scores, Sequential Organ Failure Assessment scores, and 30-day mortalities were recorded for all patients. There were no dropouts or data lost from the procedures in any manner.

Isolation of Human Monocytes

Peripheral blood mononuclear cells were isolated using Ficoll density gradient centrifugation.³² In brief, the blood was diluted with PBS. The diluted blood was then added gently to the top of the Ficoll solution (Sigma-Aldrich) and then centrifuged at 2,000 rpm for 20 min. The middle layer containing the mononuclear cells was then aspirated and washed twice with PBS. The cell pellets were resuspended in 2 ml RPMI 1640 medium (Thermo Scientific) containing 10% FBS (Moregate BioTech), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Gibco Invitrogen) and cultured in six-well plates for 2 h in a humidified atmosphere at 37°C in 5% CO₂ and 95% air. After washing the adherent monocytes three times with PBS, 1 ml TRIzol® reagent (Invitrogen, Carlsbad, CA) was added to obtain RNA.

Quantitative Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed according to one previously published research with some modifications.³³ Total RNA from monocytes of septic

patients was isolated using the TRIzol[®] reagent (Invitrogen) according to the manufacturer's instructions. Using a first-strand complementary DNA synthesis kit (Promega, Madison, WI), single-stranded complementary DNA was synthesized. Complementary DNA (1 μ l) was amplified in a 9- μ l mixture containing 5 μ l iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA), 1 μ l forward primer, 1 μ l reverse primer, and 2 μ l nuclease-free water in a rapid thermal cycler (CFX96 Real-Time System; BioRad). Each sample was run three times. After preamplification (95°C for 1 min), the samples were amplified over 40 cycles (95°C for 15 s, 58°C for 20 s, and 72°C for 20 s) with a final stop of 72°C for 5 min. All quantitative real-time polymerase chain reaction primers (table 1) were purchased from Invitrogen. Size and melting curves were performed to confirm the formation of specific polymerase chain reaction amplicons. TRPM2 and HO-1 messenger RNA (mRNA) expression in peripheral blood monocytes from septic patients (severe septic or septic shock patients) relative to controls (nonseptic patients) was analyzed using the $2^{-\Delta\Delta C_T}$ model. The mean fluorescence and threshold values (C_T) of the control gene (β -actin) were subtracted from the target gene (TRPM2 and HO-1) values to determine ΔC_T . The $\Delta\Delta C_T$ was determined by subtracting the mean ΔC_T of the control group from the septic patient group. The fold change of the target genes in septic patients relative to that in the control patients was then obtained by calculating $2^{-\Delta\Delta C_T}$.

Statistical Analyses

Data are presented as the mean \pm SD or mean \pm SEM where applicable. Differences between the two groups were analyzed using unpaired Student *t* tests. One-way ANOVAs were used to assess differences among groups, and the Bonferroni test (equal variances assumed) or Tamhane T2 test (equal variances not assumed) were used for multiple comparisons. The survival rate of septic mice was analyzed using the log-rank test. For analysis of data obtained from septic patients or nonseptic patients, we used the chi-square test to compare mortality rates between the two groups. The relations between TRPM2 and HO-1 in monocytes from septic patients were assessed using the Spearman correlation test. All data were analyzed using SPSS 17.0 for Windows (SPSS, Chicago, IL). *P* value less than 0.05 was considered

statistically significant. The sample sizes in the current study were justified based on previous works.

Results

Trpm2-KO Mice Show Decreased Survival Rate after Polymicrobial Sepsis

To investigate the overall effect of TRPM2 during polymicrobial sepsis, we assessed the survival rate of Trpm2-KO and WT mice after CLP. A CLP model with moderate lethality was generated both in Trpm2-KO and WT mice, and the survival rates were monitored for 7 days. The survival rate was 26.7% in Trpm2-KO mice and 60% in WT mice (*P* = 0.0289; fig. 1). Therefore, this finding suggests that TRPM2 plays a protective role in polymicrobial sepsis.

Trpm2-KO Mice Show Increased Bacterial Burden after Polymicrobial Sepsis

Previous study suggests that TRPM2 plays an important role in controlling *L. monocytogenes* infection.¹⁷ To evaluate whether TRPM2 plays an important role in controlling bacterial clearance in the CLP model, the bacterial burden in the blood, PLF, and vital organs was examined. At 24 h after CLP, the Trpm2-KO mice showed a significant increase in bacterial burden in the blood, PLF, liver, lung, and spleen compared with that in WT mice (fig. 2). These data suggest that TRPM2 plays an important role in controlling bacterial clearance during polymicrobial sepsis, which may contribute to its high survival rate.

Trpm2-KO Mice Show Increased Organ Injury and Systemic Inflammation after Polymicrobial Sepsis

Vital organs, such as lung and liver, are damaged during sepsis, which is associated with mortality. Hematoxylin and eosin staining was used to examine lung and liver injury. In the CLP septic mice, lung tissues showed alveolar congestion, accumulation of leukocytes, and impaired alveoli.²⁵

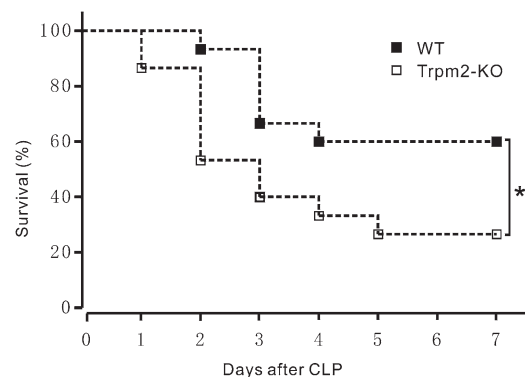


Fig. 1. Transient receptor potential melastatin 2 (TRPM2) deficiency worsens survival during cecal ligation and puncture (CLP)-induced polymicrobial sepsis. Polymicrobial sepsis was induced in wild-type (WT) and Trpm2-knockout (KO) mice by CLP surgery, and survival was monitored for 7 days (*n* = 15 per group). **P* < 0.05, Kaplan-Meier log-rank test.

Table 1. Primer Sequences for the Human Genes

Gene	Primer
β -actin	Forward primer: 5'-GTCCACCGCAAATGCTTCTA Reverse primer: 5'-TGCTGTACCTTCACCGTTC
TRPM2	Forward primer: 5'-TACTCTGCCTCTACCTGCTCTTC Reverse primer: 5'-CGTTCTTCTCCAGCTTGTCTT
HO-1	Forward primer: 5'-CTTCTCCGATGGGTCCTTACA Reverse primer: 5'-ATAGGCTCCTTCCTCCTTCC

HO-1 = heme oxygenase-1; TRPM2 = transient receptor potential melastatin 2.

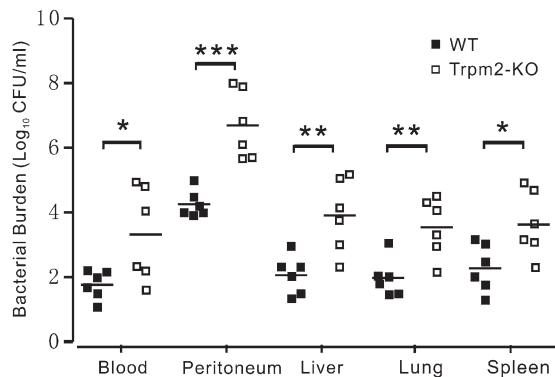


Fig. 2. Transient receptor potential melastatin 2 (TRPM2) deficiency increases bacterial burden during cecal ligation and puncture-induced polymicrobial sepsis. Wild-type (WT) and *Trpm2*-knockout (KO) mice were euthanized at 24 h after cecal ligation and puncture. Bacterial burdens were determined by plating serial dilutions of blood, peritoneal lavage fluids, or tissue homogenates on agar plates. Colony-forming units (CFUs) were counted after incubating the plates for 18 h. Each point indicates CFUs from one mouse and horizontal bars denote the means ($n = 6$ per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, Student t test.

The liver tissues of CLP mice showed increased vacuolization, cell disruption, and karyolysis.²⁶ At 24 h after CLP surgery, mice with TRPM2 gene deletion had significant lung and liver injury (fig. 3A). The lung injury indicators including lung injury scores, leukocytes infiltration, lung wet-to-dry weight ratio, and total protein concentration in bronchoalveolar lavage fluid were significantly higher in *Trpm2*-KO mice than in WT mice (fig. 3B). *Trpm2*-KO mice also have higher liver injury scores and serum alanine aminotransferase concentration compared with WT mice (fig. 3C). In addition, the serum IL-6 and high mobility group protein B1 levels were higher in *Trpm2*-KO mice than in WT mice (fig. 3D).

Disruption of TRPM2 Attenuates Lipopolysaccharide-induced HO-1 Expression in Macrophage via Decreasing Ca^{2+} Influx

Because TRPM2 is known as a redox-sensitive Ca^{2+} channel, the role of lipopolysaccharide in the channel is needed to clarify. Among BMDMs tested from WT mice, cells displayed lipopolysaccharide-induced whole cell currents with a linear current-voltage relation and a reversal potential (E_{rev}) (WT BMDM; $E_{rev} = -2.6 \pm 0.5$ mV; $n = 7$), which are characteristic of TRPM2 channels (fig. 4, A and B).^{15,27,34} In BMDMs from *Trpm2*-KO mice, this lipopolysaccharide-induced current was abolished ($n = 10$) (fig. 4, C–E). Thus, TRPM2 mediates lipopolysaccharide-induced cationic currents in murine BMDMs.

We further explored the role and mechanism of TRPM2 in bacterial clearance. A recent report suggested that HO-1 is critical for the host defense response to polymicrobial sepsis by enhancing bacterial clearance.²⁰ We first investigated

the role of TRPM2 in regulating HO-1 expression. BMDMs were cultured from *Trpm2*-KO and WT mice and were stimulated with lipopolysaccharide (control BMDMs received PBS). As expected, at 12 h after lipopolysaccharide stimulation, HO-1 expression increased in *Trpm2*-KO BMDMs and was significantly less than in WT BMDMs (fig. 4F). These data suggest that TRPM2 plays a role in controlling HO-1 expression.

We then try to explore the mechanism of TRPM2-mediated HO-1 expression. To confirm whether TRPM2-mediated Ca^{2+} influx contributes to the expression of HO-1, extracellular Ca^{2+} was removed by using different concentrations of EGTA at 30 min before lipopolysaccharide stimulation. HO-1 expression increased significantly after lipopolysaccharide treatment compared with no lipopolysaccharide treatment. However, removal of the extracellular Ca^{2+} dose-dependently reduced the increased expression of HO-1 (fig. 4G). The intracellular Ca^{2+} concentration decreased in *Trpm2*-KO BMDMs compared with WT BMDMs at 1, 2, 12, and 24 h after lipopolysaccharide stimulation (fig. 4H). These results showed that TRPM2-mediated Ca^{2+} influx may play an important role in regulation of HO-1 expression.

To further confirm the important role of TRPM2 for HO-1 expression during sepsis, we measured HO-1 expression levels in peritoneal macrophages from CLP-induced septic or sham-operated mice. At 24 h after CLP surgery, HO-1 expression was increased markedly in both WT and *Trpm2*-KO peritoneal macrophages. HO-1 levels in macrophages from *Trpm2*-KO mice were significantly lower than that of WT mice (fig. 4I).

TRPM2-mediated HO-1 Expression Is Important for Bacterial Killing in Macrophage

To investigate whether TRPM2-mediated HO-1 expression was responsible for bacterial clearance in macrophage, we performed an intracellular bacterial killing assay (fig. 5). Our results showed that disruption of TRPM2 attenuated lipopolysaccharide-induced HO-1 expression (fig. 5A). No difference was found in phagocytic capacity between WT and *Trpm2*-KO BMDMs (fig. 5B). However, the bacterial burden was greater in *Trpm2*-KO BMDMs than in WT BMDMs (fig. 5C). We next asked whether HO-1 played a role in bacterial killing. We found HO-1 inducer (hemin) could markedly increase lipopolysaccharide-induced HO-1 expression (fig. 5D) and decrease bacterial burden in the WT BMDMs compared with WT BMDMs treated with vehicle control (fig. 5F). There was no significant change in phagocytic capacity after administration of HO-1 inducer (fig. 5E). These results suggest that increased HO-1 expression improves the bacterial killing capability in macrophage. We then speculated whether disruption of TRPM2 decreased bacterial killing capability by attenuating HO-1 expression. As predicted, treatment of *Trpm2*-KO BMDMs with hemin markedly increased lipopolysaccharide-induced HO-1 expression (fig. 5G) and decreased bacterial burden

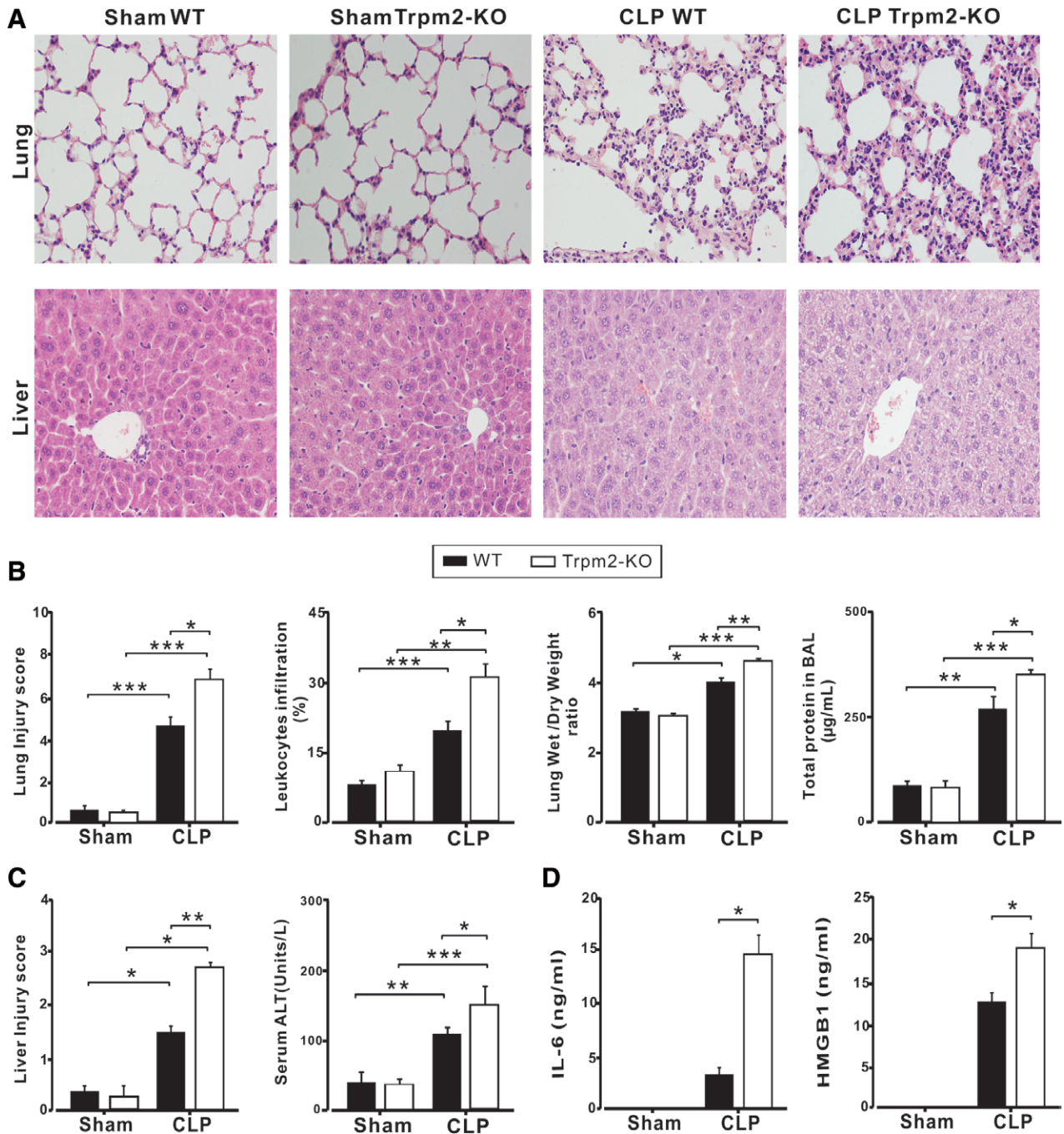


Fig. 3. Transient receptor potential melastatin 2 (TRPM2) deficiency increases organ injury during cecal ligation and puncture (CLP)-induced polymicrobial sepsis. Wild-type (WT) and Trpm2-knockout (KO) mice were euthanized at 24 h after sham or CLP. (A) Lungs and livers were harvested for hematoxylin and eosin staining (original magnifications, $\times 400$). (B) The lung injury score ($n = 6$ per group), leukocytes infiltration ($n = 6$ per group), lung wet/dry weight ratio ($n = 4$ per group), and total protein concentration in bronchoalveolar lavage (BAL) ($n = 4$ per group) represent the severity of lung injury. (C) The liver injury score ($n = 6$ per group) and serum alanine aminotransferase (ALT) concentration represent the severity of liver injury ($n = 4$ in sham group, $n = 6$ in CLP group). (D) Serum was harvested for detecting interleukin (IL)-6 ($n = 6$ in sham group, $n = 8$ in CLP group) and high mobility group protein B1 (HMGB1) ($n = 12$ per group) concentration by enzyme-linked immunosorbent assay. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA. Error bars denote the mean \pm SEM.

compared with Trpm2-KO BMDMs treated with vehicle control (fig. 5I). There was no significant change in phagocytic capacity after administration of HO-1 inducer (fig. 5H). These results suggest that TRPM2-mediated HO-1

expression may play an important role in bacterial clearance in macrophage. Disruption of TRPM2 attenuates HO-1 expression in peritoneal macrophages in CLP-induced sepsis, possibly resulting in decreased bacterial clearance. The

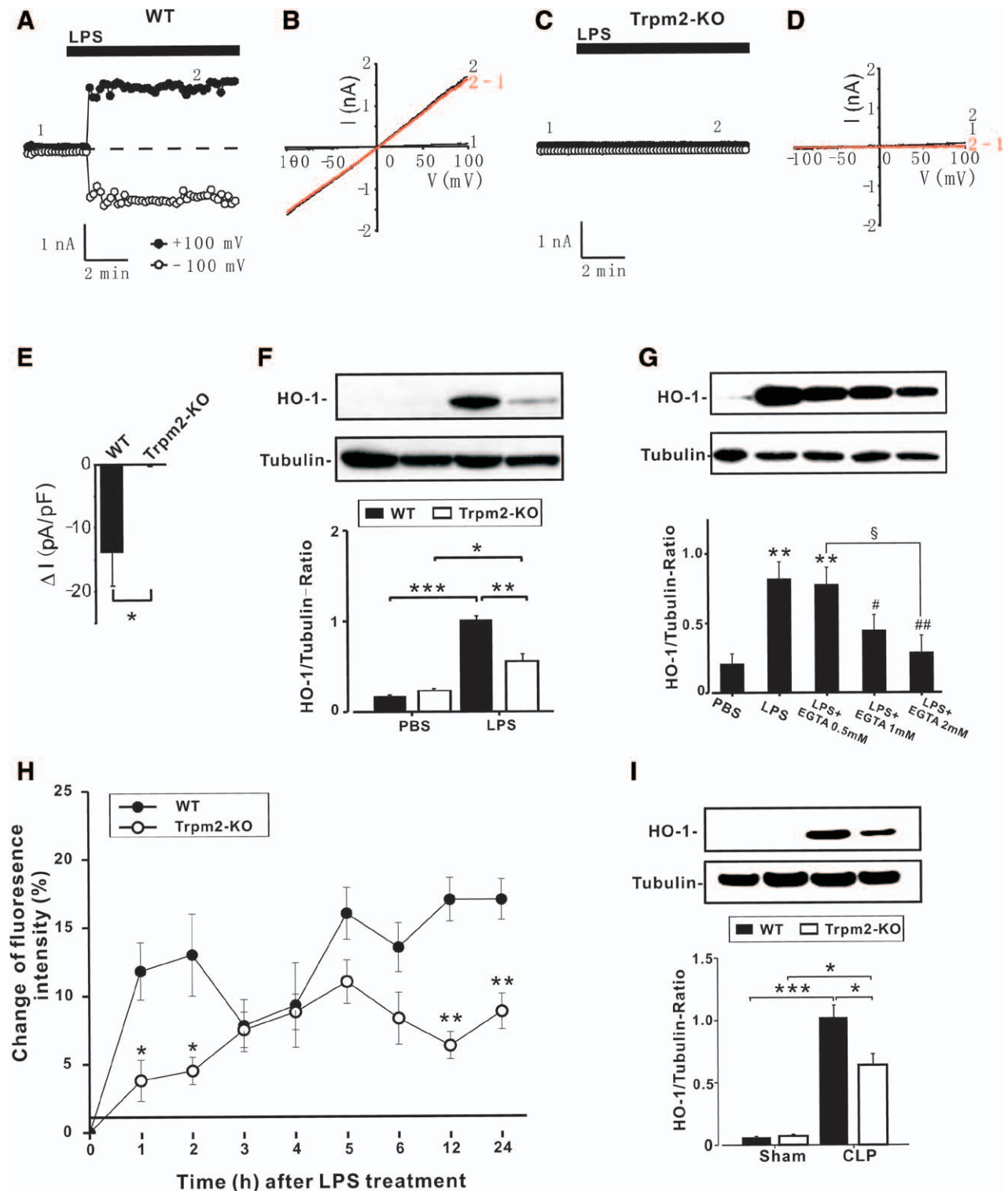


Fig. 4. Transient receptor potential melastatin 2 (TRPM2) deficiency attenuates lipopolysaccharide (LPS)-induced heme oxygenase-1 (HO-1) expression in bone marrow-derived macrophages (BMDMs) via decreasing Ca^{2+} influx. (A) Representative time courses of outward and inward whole cell currents in wild-type (WT) BMDMs with 100 ng/ml LPS stimulation. (B) Corresponding I-V relations at the time points 1 and 2 and those of induced currents (2-1) in WT BMDMs. (C) Representative time courses of outward and inward whole cell currents in Trpm2-knockout (KO) BMDMs with 100 ng/ml LPS stimulation. (D) Corresponding I-V relations at the time points 1 and 2 and those of induced currents (2-1) in Trpm2-KO BMDMs. (E) Averaged LPS-induced whole cell currents responses (ΔI) at -100 mV in WT mice ($n = 7$) and Trpm2-KO mice ($n = 10$). * $P < 0.05$, Student t test. (F) Representative gel images and densitometric quantifications of HO-1 expression in BMDMs from WT and Trpm2-KO mice with or without

increased organ injury and mortality in septic Trpm2-KO may be due to uncontrolled bacterial growth.

Pretreatment with HO-1 Inducer Increases Bacterial Clearance in WT Mice after Polymicrobial Sepsis

To confirm the role of HO-1 in controlling bacterial clearance during polymicrobial sepsis, we examined whether the HO-1 inducer improved bacterial clearance in the WT mice. We found that treatment of WT mice with hemin every other day (three times) before CLP increased HO-1 expression in peritoneal macrophages (fig. 6A) and decreased bacterial burden in the blood, PLF, liver, lung, and spleen at 24 h after CLP (fig. 6B). These results indicate that increased HO-1 expression improves the bacterial killing capability of macrophages both *in vitro* and *in vivo*.

Pretreatment with HO-1 Inducer Improves Outcome by Promoting Bacterial Clearance in Trpm2-KO Mice after Polymicrobial Sepsis

To confirm the role of TRPM2 in controlling bacterial clearance during polymicrobial sepsis *via* HO-1 expression, we next examined whether the HO-1 inducer improved bacterial clearance in the septic Trpm2-KO mice. Indeed, treatment of Trpm2-KO mice with hemin every other day (three times) before CLP increased HO-1 expression in peritoneal macrophages (fig. 7A) and decreased bacterial burden in the PLF, liver, lung, and spleen at 24 h after CLP (fig. 7B). Pretreatment of Trpm2-KO mice with hemin improved survival compared with Trpm2-KO mice treated with vehicle control ($P = 0.019$; fig. 7C).

Lung and liver injury in the Trpm2-KO mice was reversed after hemin administration (fig. 8, A–C). The serum IL-6 and HMGB1 levels were also decreased by hemin treatment

(fig. 8D). We showed that hemin, an inducer of HO-1, improves outcome by enhancing the bacterial clearance in Trpm2-KO mice. Taken together, these results suggest that TRPM2 plays an important role in controlling bacterial clearance possibly by regulating HO-1 expression and contributes to the outcome of polymicrobial sepsis.

TRPM2 Expression Correlates with HO-1 Expression in Monocytes from Septic Patients and Contributes to the Outcome

To examine whether these observations can be extended to human septic patients, we initially examined the expression of TRPM2 and HO-1 in peripheral blood monocytes collected from 25 severe septic or septic shock patients and 10 nonseptic patients. The demographics of the severe septic or septic shock patients and nonseptic patients are shown in table 2. No significant differences were found between severe septic or septic shock patients and nonseptic patients with respect to age, sex, and intensive care unit stay. Severe septic or septic shock patients had higher Acute Physiology and Chronic Health Evaluation II score ($P = 0.001$), Sequential Organ Failure Assessment score ($P = 0.014$), and 30-day mortality ($P = 0.042$) compared with nonseptic patients. In severe septic or septic shock patients, 9 (36%), 14 (56%), and 2 (8%) patients had pulmonary, abdominal, and soft-tissue infections, respectively. Initial diagnoses include pneumonia (36%), intestinal perforation (32%), pancreatic (12%), liver abscess (4%), cholecystitis (4%), ileus (4%), and multiple injuries (8%). Quantitative real-time polymerase chain reaction analysis showed that septic patients who have lower monocytic TRPM2 and HO-1 mRNA levels have a worse outcome compared with those who have normal monocytic TRPM2 and HO-1 mRNA levels (fig. 9, A and B). No differences were found in TRPM2 and HO-1 mRNA levels between nonseptic patients and recovered septic patients. To investigate whether TRPM2 correlates with HO-1, we analyzed the expression levels of TRPM2 and HO-1 using a Spearman correlation test. We observed that the TRPM2 mRNA levels were significantly correlated with HO-1 mRNA levels ($r = 0.675$, $P = 0.001$; fig. 9C).

Discussion

This study demonstrates that TRPM2 has an important role in survival, bacterial burden, organ injury, and systemic inflammation during polymicrobial sepsis. We found a protective role of TRPM2 in polymicrobial sepsis because its deletion in mice resulted in increased mortality. The increased mortality was associated with increased bacterial burden, organ injury, and systemic inflammation after CLP. The protective role of TRPM2 was possibly mediated by the HO-1. We found that HO-1 played an important role in bacterial clearance in macrophage. TRPM2-mediated Ca^{2+} influx plays an important role in lipopolysaccharide-induced HO-1 expression in macrophage. Genetic disruption of

Fig. 4. (Continued) LPS (100 ng/ml stimulation for 12 h) using Western blotting ($n = 3$ per group). The HO-1 protein concentrations were normalized by α -tubulin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA. (G) Representative gel images and densitometric quantifications of HO-1 expression in BMDMs from WT mice after pretreatment with different concentrations of EGTA for 30 min and then LPS (100 ng/ml) or no LPS stimulation for 12 h using Western blotting ($n = 3$ per group). The HO-1 protein concentrations were normalized by α -tubulin. ** $P < 0.01$ compared with phosphate-buffered saline (PBS) stimulation, # $P < 0.05$; ## $P < 0.01$ compared with LPS stimulation, and § $P < 0.01$ compared with LPS + EGTA 0.5 mM group. Data were analyzed with one-way ANOVA. (H) The time course of intracellular Ca^{2+} concentration changes in BMDMs from WT and Trpm2-KO mice after stimulation with 100 ng/ml LPS at the indicated time points. The changes in fluorescence intensity in fluo-3AM-loaded BMDMs denote LPS-induced changes in intracellular Ca^{2+} concentration ($n = 5$ per group). * $P < 0.05$; ** $P < 0.01$ compared with WT group, Student t test. (I) Representative gel images and densitometric quantifications of HO-1 expression in peritoneal macrophages from WT and Trpm2-KO mice at 24 h after sham and cecal ligation and puncture (CLP) surgery using Western blotting ($n = 5$ per group). The HO-1 protein concentration was normalized by α -tubulin. * $P < 0.05$; *** $P < 0.001$, one-way ANOVA. Error bars denote the mean \pm SEM.

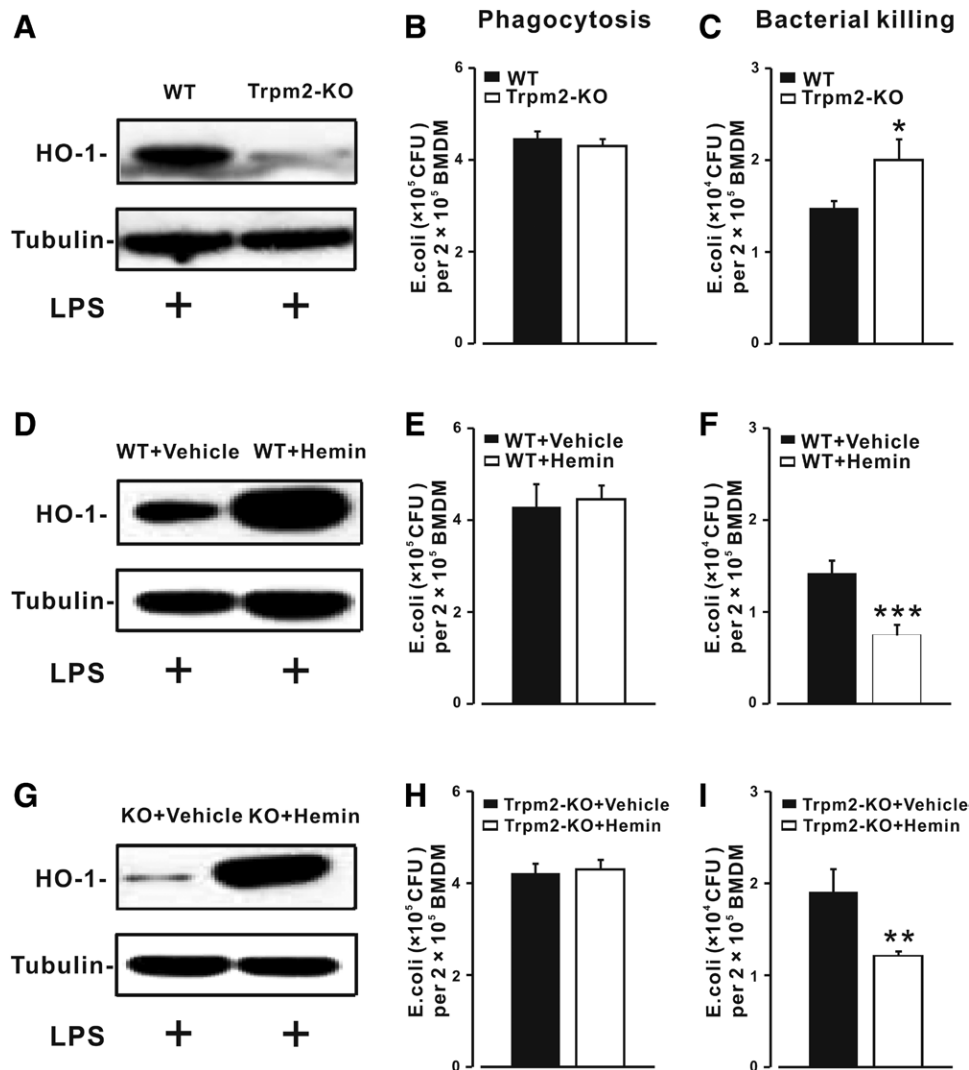


Fig. 5. Transient receptor potential melastatin 2 (TRPM2) deficiency decreases bacterial killing capability by inhibiting heme oxygenase-1 (HO-1) expression. (A) Representative gel images of HO-1 expression in bone marrow-derived macrophages (BMDMs) from wild-type (WT) and Trpm2-knockout (KO) mice with lipopolysaccharide (LPS) (100 ng/ml stimulation for 12 h) using Western blotting (n = 3 per group). (B) BMDMs from WT and Trpm2-KO mice were treated with *Escherichia coli* (DH5 α) at a multiplicity of infection (MOI) of 100 for 1 h at 37°C. Cell lysates were plated on agar plates to determine phagocytosis (n = 4 per group). (C) BMDMs from WT and Trpm2-KO mice were treated with *E. coli* at an MOI of 100 for 1 h at 37°C followed by administration of 100 μ g/ml gentamicin for 12 h. Cell lysates were plated on agar plates to determine bacterial killing capability (n = 4 per group). (D) BMDMs from WT mice were treated with vehicle or 100 μ M hemin for 6 h followed by treatment with 100 ng/ml LPS for 12 h. Representative gel images of HO-1 expression using Western blotting (n = 3 per group). (E) BMDMs from WT mice were treated with vehicle or 100 μ M hemin for 6 h followed by treatment with *E. coli* at an MOI of 100 for 1 h at 37°C. Cell lysates were plated on agar plates to determine phagocytosis (n = 4 per group). (F) BMDMs from WT mice were treated with vehicle or 100 μ M hemin for 6 h followed by treatment with *E. coli* at an MOI of 100 for 1 h at 37°C. The cells were further treated with 100 μ g/ml gentamicin for 12 h. Cell lysates were plated on agar plates to determine bacterial killing capability (n = 4 per group). (G) BMDMs from Trpm2-KO mice were treated with vehicle or 100 μ M hemin for 6 h followed by treatment with 100 ng/ml LPS for 12 h. Representative gel images of HO-1 expression using Western blotting (n = 3 per group). (H) BMDMs from Trpm2-KO mice were treated with vehicle or 100 μ M hemin for 6 h followed by treatment with *E. coli* at an MOI of 100 for 1 h at 37°C. Cell lysates were plated on agar plates to determine phagocytosis (n = 4 per group). (I) BMDMs from Trpm2-KO mice were treated with vehicle or 100 μ M hemin for 6 h followed by treatment with *E. coli* at an MOI of 100 for 1 h at 37°C. The cells were further treated with 100 μ g/ml gentamicin for 12 h. Cell lysates were plated on agar plates to determine bacterial killing capability (n = 4 per group). **P* < 0.05; ***P* < 0.01; ****P* < 0.001, Student *t* test. Error bars denote the mean \pm SEM. CFU = colony-forming units.

TRPM2 decreased lipopolysaccharide-induced HO-1 expression and increased bacterial burden in BMDMs. Pretreatment of Trpm2-KO BMDMs with HO-1 inducer

markedly increased HO-1 expression and decreased bacterial burden. Genetic disruption of TRPM2 also attenuated HO-1 expression in peritoneal macrophages and increased

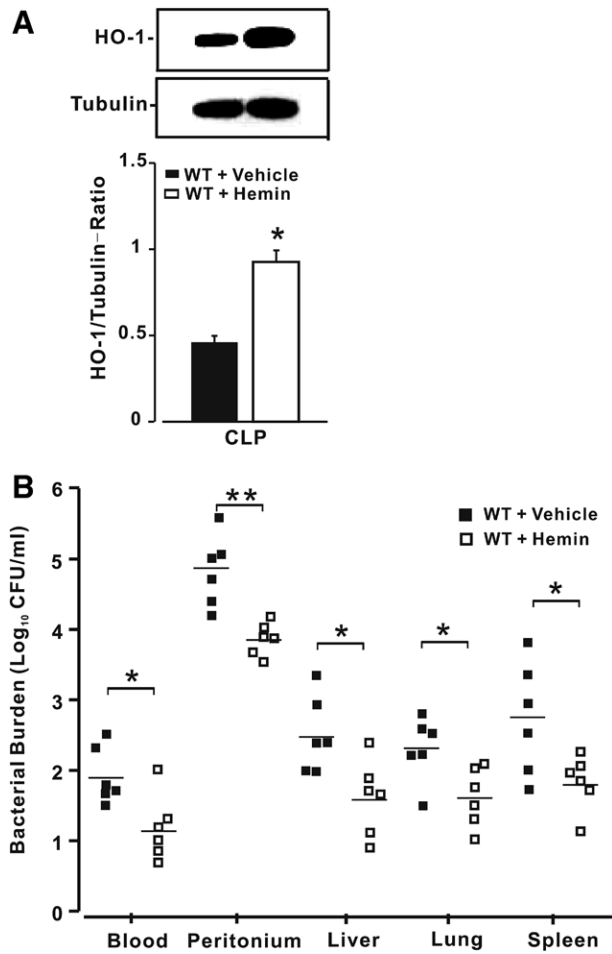


Fig. 6. The heme oxygenase-1 (HO-1) inducer increases bacterial clearance in wild-type (WT) mice after cecal ligation and puncture (CLP). The WT mice were injected with 10 mg/kg hemin or vehicle (intraperitoneal) every other day (three times) before CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. Mice were euthanized at 24 h after CLP. (A) Representative gel images and densitometric quantifications of HO-1 expression in peritoneal macrophages from WT mice using Western blotting ($n = 4$ per group). The HO-1 protein concentration was normalized by α -tubulin. $*P < 0.05$, Student t test. Error bars denote the mean \pm SEM. (B) Bacterial burdens in blood, peritoneal lavage fluids, or tissue homogenates were examined by counting colony-forming units (CFUs) ($n = 6$ per group). Each dot denotes the CFU of one mouse. $*P < 0.05$; $**P < 0.01$, Student t test. Horizontal bars denote the means.

bacterial burden in CLP-induced sepsis. Pretreatment of Trpm2-KO mice with HO-1 inducer decreased the bacterial burden and improved the outcome of septic Trpm2-KO mice. In addition, TRPM2 levels correlated with HO-1 levels in monocytes from septic patients, and severe septic patients who have low TRPM2 and HO-1 mRNA levels in monocytes have a fatal outcome. Therefore, our findings suggest a protective role of TRPM2 in controlling bacterial clearance during polymicrobial sepsis, possibly by regulating HO-1 expression.

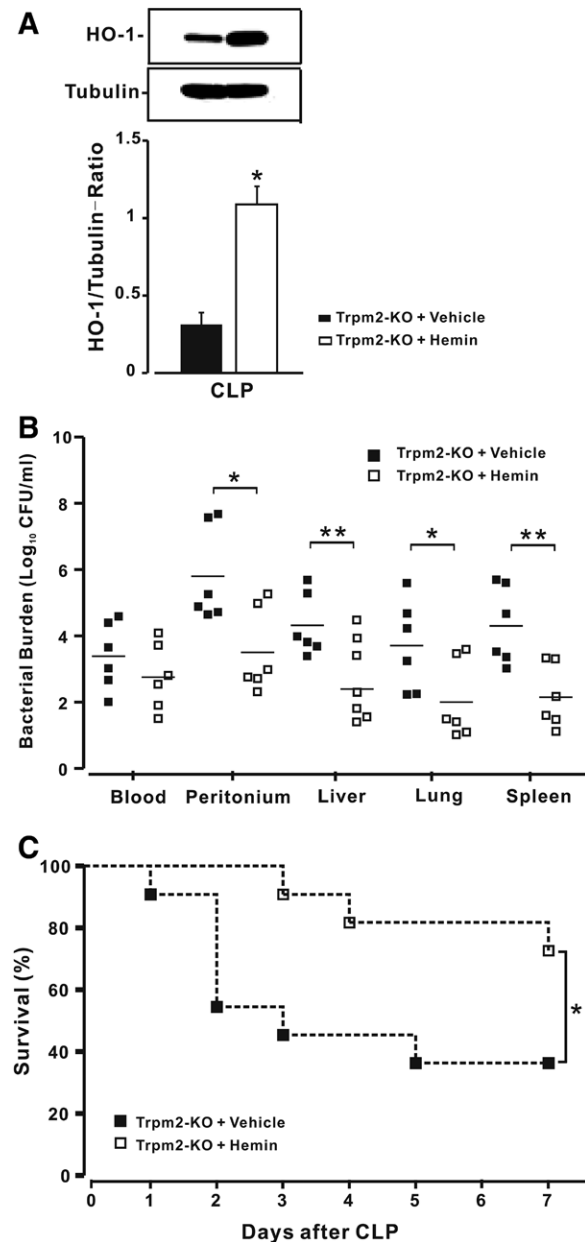


Fig. 7. The heme oxygenase-1 (HO-1) inducer improves survival by promoting bacterial clearance in transient receptor potential melastatin 2 (Trpm2)-knockout (KO) mice after cecal ligation and puncture (CLP). The Trpm2-KO mice were injected with 10 mg/kg hemin or vehicle (intraperitoneal) every other day (three times) before CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. Mice were euthanized at 24 h after CLP. (A) Representative gel images and densitometric quantifications of HO-1 expression in peritoneal macrophages from Trpm2-KO mice using Western blotting ($n = 4$ per group). The HO-1 protein concentration was normalized by α -tubulin. $*P < 0.05$, Student t test. Error bars denote the mean \pm SEM. (B) Bacterial burdens in blood, peritoneal lavage fluids, or tissue homogenates were examined by counting colony-forming units (CFUs) ($n = 6$ per group). Each dot denotes the CFU of one mouse. Horizontal bars denote the means. $*P < 0.05$; $**P < 0.01$, Student t test. (C) Survival was monitored for 7 days ($n = 11$ per group). $*P < 0.05$, Kaplan-Meier log-rank test.

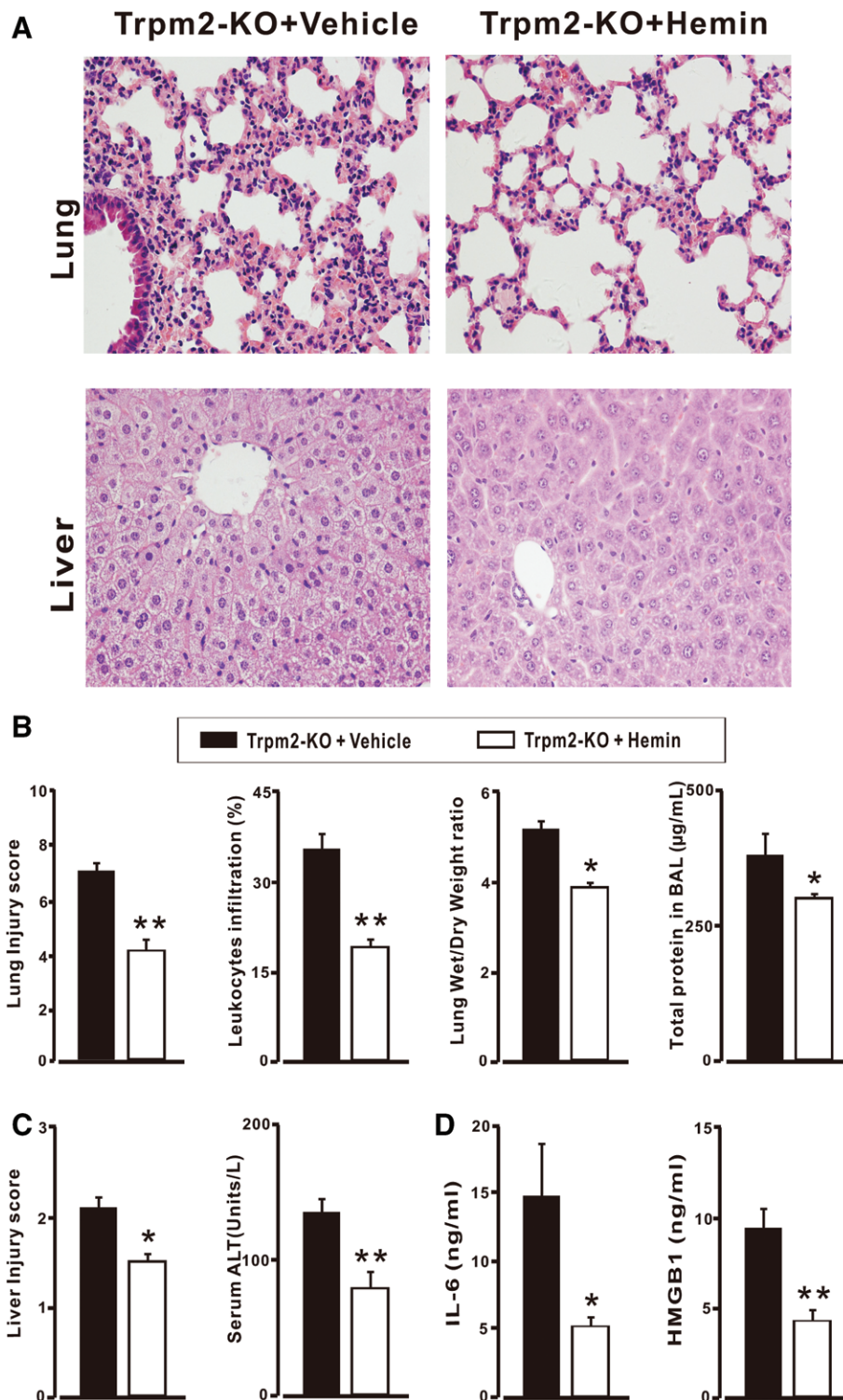


Fig. 8. The heme oxygenase-1 inducer attenuates organ injury in transient receptor potential melastatin 2 (Trpm2)-knockout (KO) mice after cecal ligation and puncture (CLP). The Trpm2-KO mice were injected with 10 mg/kg hemin or vehicle (intraperitoneal) every other day (three times) before CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. Mice were euthanized at 24 h after CLP. (A) Lungs and livers were harvested for hematoxylin and eosin staining (original magnifications, $\times 400$). (B) The lung injury score ($n = 6$ per group), leukocytes infiltration ($n = 6$ per group), lung wet/dry weight ratio ($n = 4$ per group), and total protein concentration in bronchoalveolar lavage (BAL) ($n = 4$ per group) represent the severity of lung injury. (C) The liver injury score ($n = 6$ per group) and serum alanine aminotransferase (ALT) concentration represent the severity of liver injury ($n = 4$ per group). (D) Serum was harvested for detecting interleukin (IL)-6 and high mobility group protein B1 (HMGB1) levels by enzyme-linked immunosorbent assay ($n = 6$ per group). * $P < 0.05$; ** $P < 0.01$, Student t test. Error bars denote the mean \pm SEM.

Table 2. Patient Demographics

Characteristics	Severe Sepsis or Septic Shock Patients (n = 25)	Nonseptic Patients (n = 10)	P Value
Age (yr, mean \pm SD)	60.0 \pm 16.2	61.0 \pm 9.6	0.846
Sex, male (%)	16 (64.0)	7 (70.0)	0.735
APACHE II score (mean \pm SD)	17.7 \pm 6.7	9.6 \pm 3.4	0.001
SOFA score (mean \pm SD)	7.4 \pm 3.8	4.1 \pm 2.3	0.014
Diagnosis:		NA	
Peritonitis	14 (56)		
Pneumonia	9 (36%)		
Multiple injuries	2 (8%)		
ICU stay (days, mean \pm SD)	19.5 \pm 12.5	13.1 \pm 6.3	0.137
28-day mortality, No. (%)	8 (32)	0 (0)	0.042

Data are expressed as the mean \pm SD or number (%) where applicable.

APACHE II = Acute Physiology and Chronic Health Evaluation II; ICU = intensive care unit; NA = not applicable; SOFA = Sequential Organ Failure Assessment.

Two recent reports investigated the role of TRPM2 in bacterial infusion model and lipopolysaccharide-induced endotoxin model.^{17,18} However, endotoxin models are unable to faithfully reproduce the pathogenesis of human sepsis. First, serum inflammatory cytokine levels in lipopolysaccharide-injected mice are significantly higher than in clinical septic patients. Second, the inflammatory response is entirely deleterious in endotoxemia, and the immune system is not activated because there is no focus of infection. Finally, lipopolysaccharide injection frequently causes a hypodynamic cardiovascular state, whereas the hemodynamic changes during human sepsis comprise an early hyperdynamic phase and a late hypodynamic phase.³⁵ Bacterial infusion models also lack the infection focus, and high doses of bacteria administration do not replicate within the human septic patients.³⁵ CLP-induced sepsis is characterized by a systemic inflammatory response and bacteremia with hemodynamic changes that closely mimic the clinical features of human sepsis.^{35,36} To date, the CLP model is the definitive standard model for sepsis research.^{34,36} Thus, to investigate the role of TRPM2 in sepsis, we selected the CLP model that closely reflects the pathogenesis of human sepsis.

To date, many studies have focused on the effects of TRPM2 on inflammation.^{15–17,37} Yamamoto *et al.*¹⁵ have shown that the production of chemokine (C-X-C motif) ligand 2 is impaired in monocytes from *Trpm2*-KO mice. Parallel experiments showed that chemokine (C-X-C motif) ligand 2, interferon- γ , and IL-12 release, neutrophil infiltration, and ulceration were strongly attenuated in *Trpm2*-KO mice with dextran sulfate sodium-induced colitis. TRPM2 is also required for lipopolysaccharide-induced production of IL-6, tumor necrosis factor- α , IL-8, and IL-10 in human monocytes.¹⁶ After *L. monocytogenes* infection, interferon- γ and IL-12 production is also strongly suppressed in *Trpm2*-KO mice, which represents their reduced innate activity.¹⁷ The decreased chemokine (C-X-C motif) ligand 2 and inducible nitric oxide synthase

induction in *Trpm2*-KO mice is beneficial in preventing the inflammatory and neuropathic pain.³⁷ In contrast, in a lipopolysaccharide-induced lung injury model, disruption of TRPM2 receptor augments the production of chemokine (C-X-C motif) ligand 2, IL-6, and tumor necrosis factor- α in the lung and promotes lung injury in mice.¹⁸ These discrepant roles of TRPM2 in inflammation possibly result from different models and inflammatory stimuli used. To date, the role and mechanism of TRPM2 in polymicrobial sepsis are insufficiently explored and further investigations are needed.

Although advances had made in critical care medicine and broad-spectrum antibiotics, many septic patients were unable to eradicate their infections and were more susceptible to develop secondary infections.¹⁰ Postmortem examinations also indicated that most unrecovered septic patients had unresolved foci of infection.¹⁰ Macrophages, the first line of host defense for killing invading microorganisms, play a central role in innate immunity. However, macrophage function is severely impaired in septic patient, resulting in an uncontrolled microbial growth.¹² One recent study found that mice deficient in the transient receptor potential vanilloid 1, a member of subgroup of the transient receptor potential family, also susceptible to CLP-induced sepsis possibly due to decreased bacterial clearance.³⁸ Similarly, previous study suggested that TRPM2 played an important role in controlling *L. monocytogenes* infection.¹⁷ Besides these findings, Kashio *et al.*¹⁴ also suggested that disruption of TRPM2 could impair the fever-enhanced phagocytic activity in zymosan-stimulated macrophages; then, we proposed whether TRPM2 plays an important role in controlling bacterial clearance in the CLP-induced polymicrobial sepsis. Using the CLP-induced polymicrobial sepsis model, we found that disruption of TRPM2 did result in uncontrolled bacterial growth, which may underlie the increased organ injury, increased systemic inflammation, and decreased survival after sepsis.

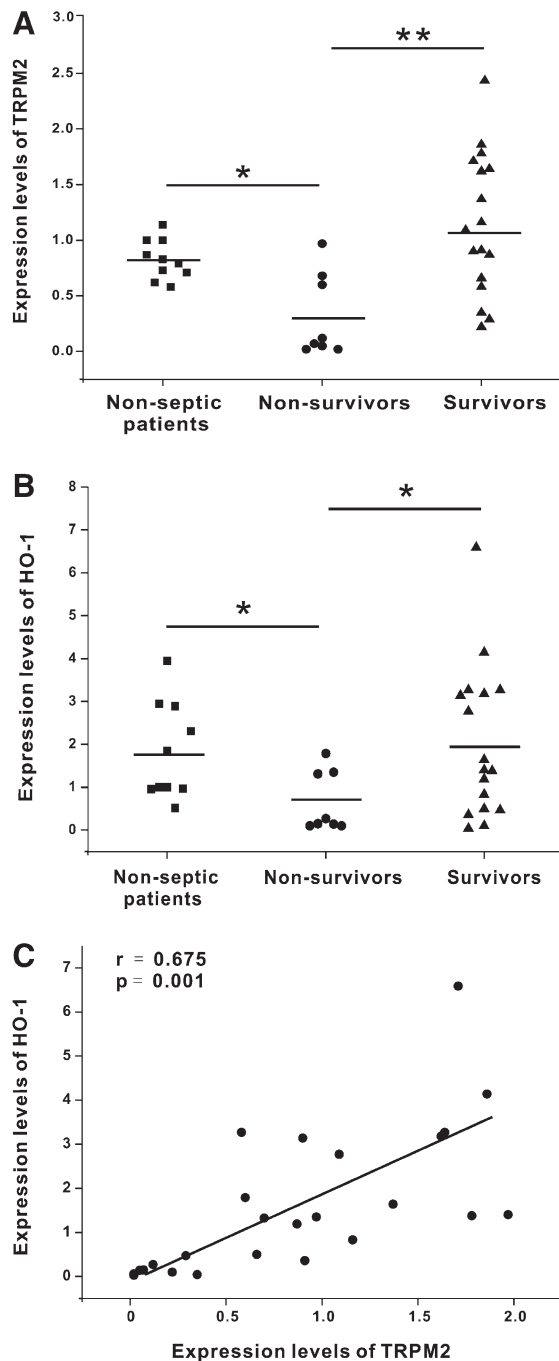


Fig. 9. Transient receptor potential melastatin 2 (TRPM2) messenger RNA (mRNA) levels correlate with heme oxygenase-1 (HO-1) mRNA levels in monocytes from septic patients and contributed to the outcome. (A and B) Monocyte TRPM2 and HO-1 mRNA levels from nonseptic patients (controls, $n = 10$) and surviving ($n = 17$) or nonsurviving ($n = 8$) severe septic or septic shock patients were examined using quantitative real-time polymerase chain reaction. TRPM2 and HO-1 mRNA was normalized to β -actin mRNA levels. Horizontal bars denote the mean values. * $P < 0.05$; ** $P < 0.01$, one-way ANOVA. (C) Correlations of TRPM2 mRNA and HO-1 mRNA ($r = 0.675$; $P = 0.001$, Spearman correlation test) in monocytes from surviving ($n = 17$) and nonsurviving ($n = 8$) severe septic or septic shock patients.

We next attempted to explore the role and the underlying mechanism of TRPM2 in bacterial clearance within macrophage. Chung *et al.*²⁰ have established the key role of HO-1 in bacterial clearance during microbial sepsis. In the CLP model, HO-1-null mice had significantly higher bacteremia and less survival rates compared with WT mice, and overexpression of HO-1 could significantly improve the bacterial clearance in circulating blood and decreased the mortality without producing an immunosuppressive disorder.²⁰ This findings provide a protective properties of HO-1 during microbial sepsis by enhancing bacterial clearance. TRPM2, an oxidative stress-activated nonselective Ca^{2+} -permeable channel, mediates Ca^{2+} entry into macrophages in response to lipopolysaccharide or H_2O_2 stimulation.^{15,16} Because Ca^{2+} influx is critical for HO-1 induction,²¹ this allowed us to propose that TRPM2-mediated Ca^{2+} influx most likely regulates HO-1 expression in macrophages. Using BMDMs from WT and *Trpm2*-KO mice, we first confirmed that lipopolysaccharide indeed activated TRPM2. We found that WT BMDMs stimulated with lipopolysaccharide displayed whole cell currents with a characteristic of TRPM2 channels.^{15,27,34} Disruption of TRPM2 abolished this lipopolysaccharide-induced current. We next observed that lipopolysaccharide-induced HO-1 expression was reduced by genetic deletion of TRPM2 or by removing extracellular Ca^{2+} . Consistent with previous studies,^{15,16} we also demonstrated that disrupting TRPM2 reduced the lipopolysaccharide-induced increase in intracellular Ca^{2+} concentration. Taken together, these results indicate that TRPM2 may acts *via* Ca^{2+} to control HO-1 expression. The HO-1 expression in peritoneal macrophages from septic *Trpm2*-KO mice was also decreased, which confirmed the role of TRPM2 in controlling HO-1 in macrophage. By performing bacterial killing experiments, we verified that TRPM2 played an important role in bacterial clearance in macrophage, possibly by regulating HO-1 expression.

The decreased expression level of HO-1 in macrophages likely underlies the decreased bacterial clearance observed in *Trpm2*-KO mice after sepsis. To confirm this hypothesis, the *Trpm2*-KO mice were injected with an inducer of HO-1 or vehicle before CLP. We observed that the *Trpm2*-KO mice pretreated with the HO-1 inducer showed a significant enhancement of bacterial clearance after CLP. Enhanced bacterial clearance after HO-1 inducer pretreatment explained the associated alleviated organ injury, decreased systemic inflammation, and improved survival after CLP. Therefore, disruption of TRPM2, possibly by decreasing the expression of HO-1, could impair the host's ability to clear bacterial infection. However, the detailed signaling pathways downstream of TRPM2-mediated HO-1 in bacterial killing are unclear. Recent researches suggested that HO-1 is critical for lipopolysaccharide-induced autophagy in macrophages.³⁹ Another more recent study showed that HO-1-mediated autophagy plays an important role in preventing liver

injury during sepsis.⁴⁰ Whether TRPM2-mediated HO-1 expression has a role in bacterial clearance by regulating autophagy in macrophages is unknown and required further investigated.

Although no human studies have explored TRPM2 expression during sepsis, poor induction of HO-1 was noted to correlate with a fatal outcome in patients with severe sepsis or septic shock.⁴¹ We also observed that septic patients who have lower monocytic TRPM2 and HO-1 mRNA levels have a fatal outcome. To further confirm that TRPM2 plays an important role in HO-1 induction, we discovered that blood monocyte TRPM2 expression was significantly correlated with HO-1 expression. Reduced response of monocytes to pathogens or lipopolysaccharide stimulation coupled with impaired inflammatory cytokine production indicates immunosuppression status in severe septic patients.¹⁰ The immunosuppression compromises the host's ability to fight against pathogens and contributes to secondary infection.¹⁰ TRPM2, a calcium channel plays a critical role in controlling inflammatory cytokine production and HO-1 expression which are important for host to combat invading bacteria, is down-regulated in uncovered septic patients. TRPM2 might serve as a biomarker for identification of the immune state and as a target for immunoadjuvant therapy in severe septic or septic shock patients.

In summary, our study identifies a protective role of TRPM2 in host defense against polymicrobial sepsis by enhancing bacterial killing capacity, and this protection is possibly mediated by HO-1. Antiinflammatory treatments have shown little success in improving the survival of septic patients, indicating that antiinflammatory strategies may result in inadequate host defense against bacterial infection. New strategies aimed at improving protective immunity against bacterial infection may help in curing sepsis. The novel correlation between TRPM2 and HO-1 may be important for regulating macrophage function. Our data provide additional insight into the role and mechanism of TRPM2 in the pathogenesis of sepsis, and immunomodulatory intervention *via* TRPM2 may help in the treatment of sepsis.

Acknowledgments

The authors thank Qiang Fang, M.D., Department of Intensive Care Unit, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, for providing the patients' blood samples.

This work was funded by Key Program grant 81130036 (to Dr. Fang) from the National Natural Science Foundation of China, Beijing, People's Republic of China, and grant-in-aid (grant no. 24249017 to Dr. Mori) for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Tokyo, Japan.

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Fang: Department of Anesthesiology, the First Affiliated Hospital, College of Medicine, Zhejiang University, Qingchun Road 79, Hangzhou 310003, China. xiangming_fang@163.com; or to Dr. Mori: Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Kyoto 615-8510, Japan. mori@sbchem.kyoto-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Cohen J: The immunopathogenesis of sepsis. *Nature* 2002; 420:885–91
2. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001; 29:1303–10
3. Martin GS, Mannino DM, Eaton S, Moss M: The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003; 348:1546–54
4. van den Berg JW, van der Zee M, de Bruin RW, van Holten-Neelen C, Bastiaans J, Nagtzaam NM, IJzermans JN, Benner R, Dik WA: Mild *versus* strong anti-inflammatory therapy during early sepsis in mice: A matter of life and death. *Crit Care Med* 2011; 39:1275–81
5. Opal SM, Fisher CJ Jr, Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA, Shelly MP, Pribble JP, LaBrecque JF, Lookabaugh J, Donovan H, Dubin H, Baughman R, Norman J, DeMaria E, Matzel K, Abraham E, Seneff M: Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: A phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. *Crit Care Med* 1997; 25:1115–24
6. Fisher CJ Jr, Dhainaut JF, Opal SM, Pribble JP, Balk RA, Slotman GJ, Iberti TJ, Rackow EC, Shapiro MJ, Greenman RL: Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. *JAMA* 1994; 271:1836–43
7. Fisher CJ Jr, Agosti JM, Opal SM, Lowry SF, Balk RA, Sadoff JC, Abraham E, Schein RM, Benjamin E: Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group. *N Engl J Med* 1996; 334:1697–702
8. Reinhart K, Karzai W: Anti-tumor necrosis factor therapy in sepsis: Update on clinical trials and lessons learned. *Crit Care Med* 2001; 29(7 suppl):S121–5
9. Opal SM, Laterre PF, Francois B, LaRosa SP, Angus DC, Mira JP, Wittebole X, Dugernier T, Perrotin D, Tidswell M, Jauregui L, Krell K, Pachel J, Takahashi T, Peckelsen C, Cordasco E, Chang CS, Oeyen S, Aikawa N, Maruyama T, Schein R, Kalil AC, Van Nuffelen M, Lynn M, Rossignol DP, Gogate J, Roberts MB, Wheeler JL, Vincent JL; ACCESS Study Group: Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: The ACCESS randomized trial. *JAMA* 2013; 309:1154–62
10. Hotchkiss RS, Monneret G, Payen D: Immunosuppression in sepsis: A novel understanding of the disorder and a new therapeutic approach. *Lancet Infect Dis* 2013; 13:260–8
11. Angus DC, van der Poll T: Severe sepsis and septic shock. *N Engl J Med* 2013; 369:840–51
12. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. *N Engl J Med* 2003; 348:138–50

13. Clapham DE: TRP channels as cellular sensors. *Nature* 2003; 426:517–24
14. Kashio M, Sokabe T, Shintaku K, Uematsu T, Fukuta N, Kobayashi N, Mori Y, Tominaga M: Redox signal-mediated sensitization of transient receptor potential melastatin 2 (TRPM2) to temperature affects macrophage functions. *Proc Natl Acad Sci U S A* 2012; 109:6745–50
15. Yamamoto S, Shimizu S, Kiyonaka S, Takahashi N, Wajima T, Hara Y, Negoro T, Hiroi T, Kiuchi Y, Okada T, Kaneko S, Lange I, Fleig A, Penner R, Nishi M, Takeshima H, Mori Y: TRPM2-mediated Ca^{2+} influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. *Nat Med* 2008; 14:738–47
16. Wehrhahn J, Kraft R, Harteneck C, Hauschildt S: Transient receptor potential melastatin 2 is required for lipopolysaccharide-induced cytokine production in human monocytes. *J Immunol* 2010; 184:2386–93
17. Knowles H, Heizer JW, Li Y, Chapman K, Ogden CA, Andreasen K, Shapland E, Kucera G, Mogan J, Humann J, Lenz LL, Morrison AD, Perraud AL: Transient receptor potential melastatin 2 (TRPM2) ion channel is required for innate immunity against *Listeria monocytogenes*. *Proc Natl Acad Sci U S A* 2011; 108:11578–83
18. Di A, Gao XP, Qian F, Kawamura T, Han J, Hecquet C, Ye RD, Vogel SM, Malik AB: The redox-sensitive cation channel TRPM2 modulates phagocyte ROS production and inflammation. *Nat Immunol* 2012; 13:29–34
19. Soares MP, Bach FH: Heme oxygenase-1: From biology to therapeutic potential. *Trends Mol Med* 2009; 15:50–8
20. Chung SW, Liu X, Macias AA, Baron RM, Perrella MA: Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* 2008; 118:239–47
21. Tsoyi K, Jang HJ, Kim JW, Chang HK, Lee YS, Pae HO, Kim HJ, Seo HG, Lee JH, Chung HT, Chang KC: Stimulation of $\alpha 7$ nicotinic acetylcholine receptor by nicotine attenuates inflammatory response in macrophages and improves survival in experimental model of sepsis through heme oxygenase-1 induction. *Antioxid Redox Signal* 2011; 14:2057–70
22. Mei SH, Haitma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ: Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010; 182:1047–57
23. Nakamichi I, Habtezion A, Zhong B, Contag CH, Butcher EC, Omary MB: Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis *via* heme oxygenase-1 induction. *J Clin Invest* 2005; 115:3007–14
24. Chen Q, Zhang K, Jin Y, Zhu T, Cheng B, Shu Q, Fang X: Triggering receptor expressed on myeloid cells-2 protects against polymicrobial sepsis by enhancing bacterial clearance. *Am J Respir Crit Care Med* 2013; 188:201–12
25. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM: Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest* 2002; 110:1703–16
26. He S, Atkinson C, Qiao F, Cianflone K, Chen X, Tomlinson S: A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice. *J Clin Invest* 2009; 119:2304–16
27. Hara Y, Wakamori M, Ishii M, Maeno E, Nishida M, Yoshida T, Yamada H, Shimizu S, Mori E, Kudoh J, Shimizu N, Kurose H, Okada Y, Imoto K, Mori Y: LTRPC2 Ca^{2+} -permeable channel activated by changes in redox status confers susceptibility to cell death. *Mol Cell* 2002; 9:163–73
28. Piegeler T, Votta-Velis EG, Liu G, Place AT, Schwartz DE, Beck-Schimmer B, Minshall RD, Borgeat A: Antimetastatic potential of amide-linked local anesthetics: Inhibition of lung adenocarcinoma cell migration and inflammatory Src signaling independent of sodium channel blockade. *ANESTHESIOLOGY* 2012; 117:548–59
29. Liu B, Wang P, Wang Z, Zhang W: The use of anti-VDAC2 antibody for the combined assessment of human sperm acrosome integrity and ionophore A23187-induced acrosome reaction. *PLoS One* 2011; 6:e16985
30. Houghton AM, Hartzell WO, Robbins CS, Gomis-Rüth FX, Shapiro SD: Macrophage elastase kills bacteria within murine macrophages. *Nature* 2009; 460:637–41
31. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G: 2001 SCCM/ESICM/ACCP/ATS/SIS International sepsis definitions conference. *Crit Care Med* 2003; 29:530–8
32. Cai M, Chen Q, Chen C, Liu X, Hou J, Zeng C, Shu Q, Fang X: Activation of triggering receptor expressed on myeloid cells-1 protects monocyte from apoptosis through regulation of myeloid cell leukemia-1. *ANESTHESIOLOGY* 2013; 118:1140–9
33. Suzuki M, Narita M, Hasegawa M, Furuta S, Kawamata T, Ashikawa M, Miyano K, Yanagihara K, Chiwaki F, Ochiya T, Suzuki T, Matoba M, Sasaki H, Uezono Y: Sensation of abdominal pain induced by peritoneal carcinomatosis is accompanied by changes in the expression of substance P and μ -opioid receptors in the spinal cord of mice. *ANESTHESIOLOGY* 2012; 117:847–56
34. Numata T, Sato K, Christmann J, Marx R, Mori Y, Okada Y, Wehner F: The ΔC splice-variant of TRPM2 is the hypertonicity-induced cation channel in HeLa cells, and the ecto-enzyme CD38 mediates its activation. *J Physiol* 2012; 590(Pt 5):1121–38
35. Buras JA, Holzmann B, Sitkovsky M: Animal models of sepsis: Setting the stage. *Nat Rev Drug Discov* 2005; 4:854–65
36. Schultz MJ, van der Poll T: Animal and human models for sepsis. *Ann Med* 2002; 34:573–81
37. Haraguchi K, Kawamoto A, Isami K, Maeda S, Kusano A, Asakura K, Shirakawa H, Mori Y, Nakagawa T, Kaneko S: TRPM2 contributes to inflammatory and neuropathic pain through the aggravation of pronociceptive inflammatory responses in mice. *J Neurosci* 2012; 32:3931–41
38. Guptill V, Cui X, Khaibullina A, Keller JM, Spornick N, Mannes A, Iadarola M, Quezado ZM: Disruption of the transient receptor potential vanilloid 1 can affect survival, bacterial clearance, and cytokine gene expression during murine sepsis. *ANESTHESIOLOGY* 2011; 114:1190–9
39. Waltz P, Carchman EH, Young AC, Rao J, Rosengart MR, Kaczorowski D, Zuckerbraun BS: Lipopolysaccharide induces autophagic signaling in macrophages *via* a TLR4, heme oxygenase-1 dependent pathway. *Autophagy* 2011; 7:315–20
40. Carchman EH, Rao J, Loughran PA, Rosengart MR, Zuckerbraun BS: Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice. *Hepatology* 2011; 53:2053–62
41. Takaki S, Takeyama N, Kajita Y, Yabuki T, Noguchi H, Miki Y, Inoue Y, Nakagawa T, Noguchi H: Beneficial effects of the heme oxygenase-1/carbon monoxide system in patients with severe sepsis/septic shock. *Intensive Care Med* 2010; 36:42–8