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Inhibition of autophagy protects against sepsis by concurrently attenuating the cytokine storm and vascular leakage

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SUMMARY

Objectives: Sepsis is an overwhelming systemic inflammatory response for which no satisfactory therapeutic drug is available. Previous studies have shown that autophagy is involved in the cytokine storm and vascular leakage that occur during sepsis. Therefore, we aimed to evaluate the therapeutic potential of autophagy inhibitors against bacterial infection-induced sepsis.
Methods: Cytokine production and phagocytosis of bacteria by human leukocytes and the permeability of endothelial cells were determined after the co-incubation of cells with lipopolysaccharide (LPS) or *Escherichia coli* in the presence or absence of autophagy inhibitors *in vitro*. Furthermore, the therapeutic effects of the autophagy inhibitors in *E. coli*-infected mice were analysed.
Results: In the presence of the autophagy inhibitors, the LPS-triggered cytokine secretion of human leucocytes and LPS (or LPS-conditioned medium from leucocytes)-induced endothelial hyperpermeability were significantly reduced. Moreover, the inhibition of autophagy enhanced the clearance of *E. coli* by leucocytes *in vitro*. Finally, we demonstrated that post-treatment but not pretreatment with an autophagy inhibitor (hydroxychloroquine) completely protected mice against *E. coli* infection-induced lethality by simultaneously reducing cytokine production and vascular leakage and enhancing bacterial clearance.
Conclusions: These results suggest that autophagy plays an important role in the pathogenesis of sepsis and could serve as a potential therapeutic target for sepsis.

Introduction

Sepsis is a syndrome caused by an overwhelming systemic inflammatory response to infection which can lead to life-threatening septic shock.[1,2] Since there is no effective drug to suppress the excessive inflammatory response, sepsis is the leading cause of death in hospital intensive care units.[3–5] Hence, an effective therapeutic strategy preventing sepsis-induced lethality is in great demand.

Cytokine storms and vascular leakage are two major pathological hallmarks of sepsis.[6–10] The exposure of immune cells to the Gram-negative bacterial cell wall component lipopolysaccharide (LPS) can induce the production and secretion of many pro-inflammatory cytokines, such as tumour necrosis factor α (TNFα), interleukin 1 (IL-1), IL-6, macrophage migration inhibitory factor (MIF) and high mobility group box 1 protein (HMGB1), in septic patients.[11,12] In addition, LPS and LPS-induced cytokines can increase endothelial permeability, leading to multiple organ failure.[9,10,13] Previous studies have shown that autophagy is involved in the regulation of endothelial permeability during inflammation.[13–16] In addition, autophagy plays important roles in regulating the production, processing, and secretion of cytokines, such as IL-1.[17] Therefore, we proposed and tested the hypothesis that the modulation of autophagy may represent a potential therapeutic strategy for preventing the cytokine storm and vascular leakage that occur during sepsis.

Autophagy is a conserved homeostatic process occurring in cells in response to stress conditions.[18,19] Autophagy signalling can be induced by starvation, reactive oxygen species (ROS), endoplasmic reticulum stress and infection. The formation of autophagic vesicles requires many autophagy-related (Atg) genes. This process begins with the lipidation of the Atg8 protein (light-chain 3 I, LC3-I) by phosphatidylethanolamine to form an LC3-II conjugate. The conversion of LC3-I to LC3-II leads to the formation of a double-membrane-bound autophagosome. During the late stage of autophagy, autophagosomes fuse with lysosomes to form autophagolysosomes, and the cargos contained in the autophagosomes are then degraded by lysosomal enzymes, such as hydrolases.[20] Therefore, it is generally believed that autophagy plays a protective role in sepsis by degrading damaged proteins, killing

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pathogens, preserving mitochondrial function and preventing cell apoptosis.[21,22] However, some studies note that macrophages deficient in autophagy exhibit increased uptake of bacteria and protect mice against bacterial infection.[23,24] In addition, the inhibition of autophagy by chloroquine can either worsen or protect mice against caecum ligation and puncture-induced lethal sepsis.[25,26] Therefore, the roles of autophagy in the pathogenesis of sepsis remain controversial.

In this study, we used LPS stimulation and *Escherichia coli* infection as models to investigate the effect of autophagy inhibitors on cytokine secretion and bacterial clearance by leucocytes and the permeability of endothelial cells. We demonstrated that LPS-induced cytokine production by leucocytes was attenuated by the inhibition of autophagy. Moreover, the treatment with the autophagy inhibitors enhanced bacterial clearance by leucocytes. In addition, the endothelial hyperpermeability induced by either LPS or LPS-conditioned medium was also alleviated in the presence of the autophagy inhibitors. Finally, the inhibition of autophagy improved survival in mice by reducing both cytokine secretion and vascular leakage and increasing bacterial clearance after lethal *E. coli* infection.

Materials and methods

Ethics statement

All research involving healthy adult donors (5 healthy adult donors were recruited for this study) was approved by the National Cheng Kung University (NCKU) Hospital Institutional Review Board (IRB #A-ER-104-368). Informed written consent was obtained from the volunteers following the human experimentation guidelines of the Institutional Review Board of NCKU Hospital.

All animal studies were performed in compliance with the Guide for the Care and Use of Laboratory Animals (The Chinese-Taipei Society of Laboratory Animal Sciences, 2010) and approved by the Institutional Animal Care and Use Committee (IACUC) of NCKU under the number IACUC 105,018.

Inhibitors and treatment

In the *in vitro* cell culture experiments, the cells were co-treated with LPS and different inhibitors. Autophagy initiation was inhibited by co-treating with a PI3K inhibitor (3-methyladenine, 3-MA) (Sigma-Aldrich, St. Louis, MO) at a concentration of 2 mM or an ROS scavenger (N-acetyl-L-cysteine, NAC) (Sigma-Aldrich) at a concentration of 20 mM. The autophagosome and lysosome fusion was blocked by co-treatment with 25 nM bafilomycin A1 (BafA1, Alfa Aesar, Ward Hill, MA) or 100 μM chloroquine (CQ, Sigma-Aldrich). Neither cytotoxicity nor bactericidal activity against *E. coli* was observed at the concentration of autophagy inhibitors used. Polymyxin B (PMB) (Sigma-Aldrich) was used to neutralize LPS activity in a comparison group. In the *in vivo* experiments, a derivative of CQ, hydroxychloroquine (HCQ, Sigma-Aldrich) (65 mg/kg) and 3-MA (40 mg/kg) were intraperitoneally injected into mice to evaluate their therapeutic effects.

Isolation of peripheral blood leucocytes

Peripheral blood was collected from healthy donors using ethylenediamine tetraacetic acid (EDTA)-coated vacutainer tubes (BD Biosciences, Franklin Lakes, NJ). The leucocyte buffy coat was isolated by centrifugation at 1500 rpm for 15 min. After transferring to a sterile centrifuge tube, the buffy coat was washed with RBC lysis buffer (Sigma-Aldrich) to eliminate the erythrocytes and centrifuged at 2500 × g for 5 min at room temperature (RT). Then, the supernatant was discarded, and the pellets were washed once with phosphate-buffered saline (PBS) by centrifugation at 1500 × g for 5 min. The leucocytes were re-suspended in the desired volume of serum-free RPMI-1640 medium.

Cell culture

The human microvascular endothelial cell line (HMEC-1) (obtained from the Centres for Disease Control and Prevention, CDC, Taiwan) and human umbilical vein endothelial cells (HUVECs) (Bioresource Collection and Research Centre, BCRC, Taiwan) were cultured in Medium 200 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; HyClone Laboratories, Logan, UT). The human monocytic cell line (THP-1) was grown in RPMI-1640 medium (Life Technologies, Carlsbad, CA) containing 10% FBS, 2 mM L-glutamine (Caisson Laboratories, Smithfield, UT, USA) and 50 μg/ml gentamycin (MDBio, Taiwan). Stable clones of luciferase (Luc) knockdown HMEC-1 cells, GFP knockdown THP-1 cells and LC3 knockdown THP-1 cells were generated by a lentivirus-based short hairpin RNA (shRNA) system (National RNAi Core Facility, Academia Sinica, Taiwan). The Atg5-silenced HMEC-1 cells were a generous gift from Dr. Chiou-Feng Lin (Department of Microbiology and Immunology, Taipei Medical University). The HMEC-1 and THP-1 stable clones were selected with 250 ng/ml or 2 μg/ml puromycin (MDBio), respectively. All cells were cultured in a 37°C incubator in a humidified 5% CO_2 atmosphere.

LPS stimulation of leucocytes and THP-1 cells

Leucocytes (2.5×10^5 cells/well) were seeded in 24-well plates and stimulated with 1 μg/ml LPS (Sigma-Aldrich) or not stimulated. The cell culture medium was collected 24 h after the stimulation and centrifuged at 1000 × g for 6 min to obtain the supernatant for the cytokine measurement or storage as the LPS-conditioned medium or normal control (NC) medium. The THP-1 cells (1×10^6 cells/ml) were exposed to 5 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h to induce differentiation. The PMA-differentiated THP-1 cells (2.5×10^5 cells/well) and leucocytes were stimulated with 1 μg/ml LPS in the presence or absence of autophagy inhibitors. The cell culture medium was collected at the indicated time points after treatment and centrifuged at 1000 × g for 6 min to obtain the supernatant for the cytokine measurement.

Cytokine measurement

The concentration of human IL-1β, IL-6, MIF and HMGB1 in the cell culture medium or mouse plasma was measured using human or mouse ELISA kits following the manufacturers' instructions. The human and mouse IL-1β ELISA kits were purchased from Invitrogen (Carlsbad, CA) and BioLegend (San Diego, CA), respectively. The human and mouse IL-6 and MIF ELISA kits were purchased from R&D Systems (Minneapolis, MN). The human and mouse HMGB1 ELISA kits were purchased from Chondrex, Inc. (Redmond, WA) and Leadgene (Taiwan), respectively.

Endothelial permeability assay

HMEC-1 cells (9×10^4/well) were seeded in the upper chambers of Transwell plates (0.4 μm; Corning B.V. Life Sciences, The Netherlands) and maintained in M200 medium containing 10% FBS for 5 days. After the cells formed a stable monolayer, LPS or LPS-conditioned medium with or without the inhibitors was added to the upper chambers along with M200 medium. After 1 or 6 h of treatment as indicated, the medium in the upper chambers was replaced with 300 μl of RPMI medium containing 4.5 μl of streptavidin-horseradish peroxidase (HRP) (R&D Systems). The

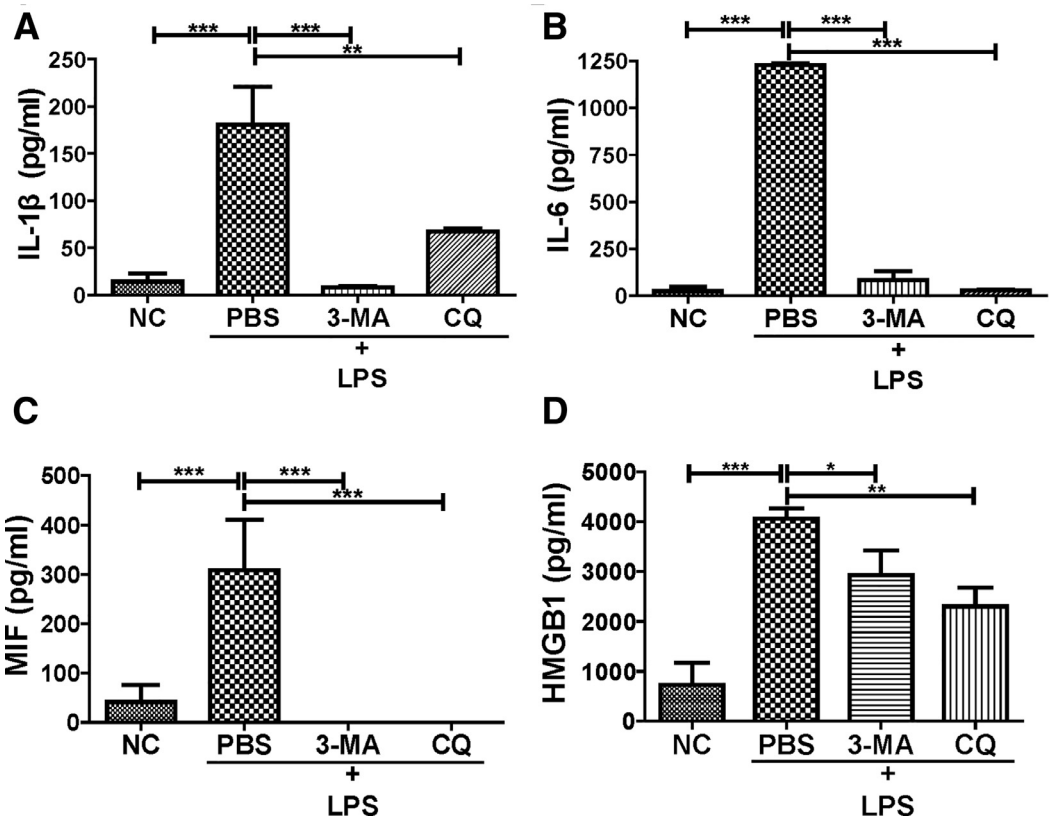

Fig. 1. Autophagy inhibitors suppressed LPS-induced cytokine secretion in human leucocytes. Isolated human leucocytes were co-treated with LPS (1 μg/ml) with or without autophagy inhibitors (3-MA or CQ) for 24 h, and PBS only treatment was used as normal control ($n = 4$ per group). Cell supernatants were collected, and the concentrations of **(A)** IL-1β, **(B)** IL-6, **(C)** MIF and **(D)** HMGB1 in the cell supernatants were determined by ELISA kits. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

streptavidin-HRP was allowed to equilibrate for 10 min, 50 μl of the medium in the lower chambers were collected, and HRP activity was assayed by adding 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate (R&D Systems). The colour development was detected by a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at OD $_{450\ nm}$.

Western blotting

The proteins were separated using SDS-PAGE and transferred onto a PVDF membrane (Pall, Ann Arbor, MI, USA). The membrane was blocked with 5% skim milk in TBST (0.05% Tween 20 in TBS) for 1 h. Then, the membrane was incubated with antibodies against LC3 (GeneTex, Irvine, CA) or β-actin (Sigma-Aldrich) at 4°C overnight and washed with TBST. Then, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit immunoglobulin secondary antibodies (1:10,000 dilution) (Leadgene) for another 1 h. After washing with TBST, the bound HRP-conjugated antibodies were detected by WesternBright ECL reagents (Advansta, Menlo Park, CA). The Western blotting results were quantitatively analysed using ImageJ software.

Preparation of E. coli

E. coli isolated from the blood of patients with sepsis was a generous gift from Dr. Pei-Jane Tsai (Department of Medical Laboratory Science and Biotechnology, NCKU). *E. coli* was maintained on LB agar plates for 16 h. Before each test, a loopful of the bacteria from the plate was transferred into 10 ml of sterile LB broth and grown for 16-20 h at 37°C and 100 rpm. Then, the *E. coli*-containing medium was washed with sterile PBS to eliminate the LB broth by centrifugation at 3000 × g for 5 min at RT. After the supernatant was discarded, the pellet was re-suspended in 1 ml of PBS, and the concentration of *E. coli* was determined by spectrometry at OD $_{600\ nm}$. The *E. coli* stock was adjusted to the desired concentration by using PBS.

Phagocytosis assay

Leucocytes (4×10^5 cells/tube) were infected with *E. coli* (at a multiplicity of infection of 10:1) and co-treated with or without autophagy inhibitors in cell culture tubes for 2 h at 37°C with shaking. Then, the cell culture tubes were centrifuged at 100 × g for 10 min to separate the cells from the supernatants. The infected cells were washed once with PBS and treated with gentamycin (125 μg/ml) for 3 h to eliminate the residual extracellular bacteria. After washing with PBS, the cells were lysed in 200 μl of distilled water for 15 min, and the intracellular bacteria were harvested. The extracellular bacteria in the supernatants and the intracellular bacteria in the cell lysates were serially diluted, and 30 μl of each sample were plated on LB agar plates. After 18 to 22 h of culture at 37°C, the bacterial load was calculated by counting the colony-forming units (CFU).

Bacterial sepsis models and treatment

Eight- to ten-week-old female BALB/c mice were purchased from the National Laboratory Animal Centre and maintained at the Laboratory Animal Centre of NCKU. The mice were weighed and divided into the following four groups ($n = 6$ per group): Group 1 (control, intraperitoneal (i.p.) injection of PBS only), Group 2 (*E. coli* infection, i.p. injection of *E. coli* (5×10^9 CFU/kg) in PBS), Group 3 (post-treatment with 3-MA, i.p. injection of 3-MA (40 mg/kg) 1.5 h after the *E. coli* infection), and Group 4 (post-treatment with HCQ,

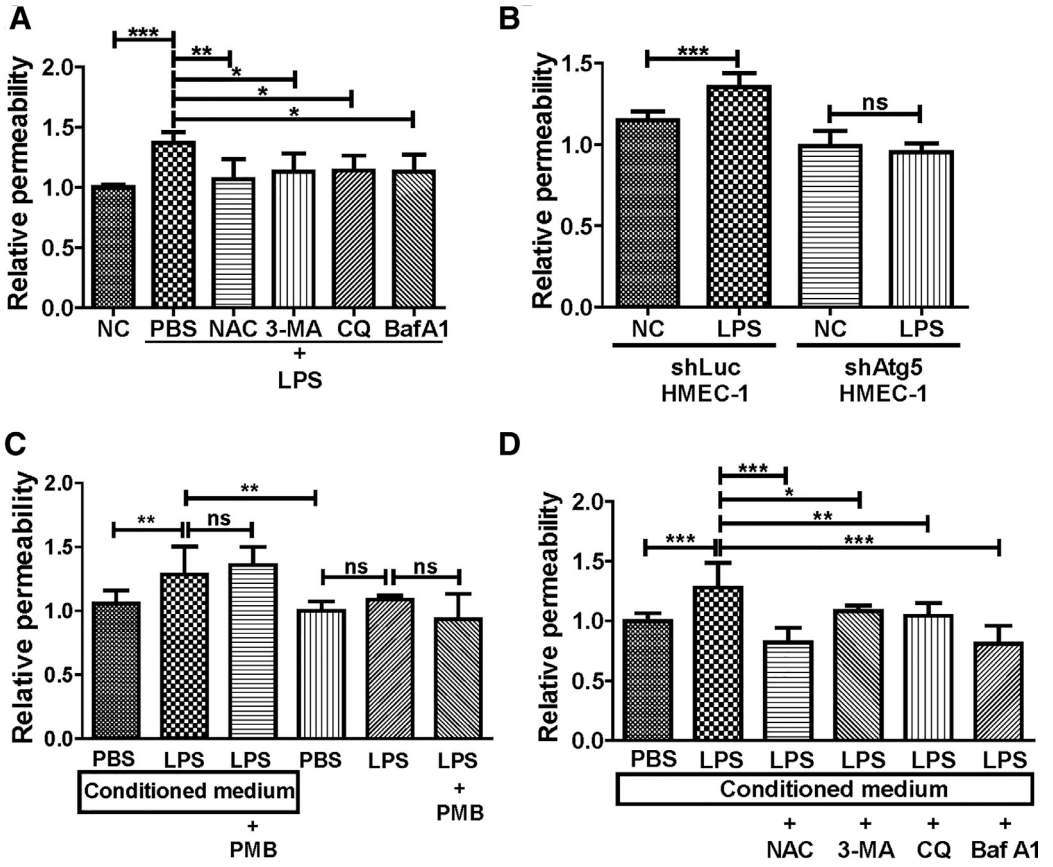

Fig. 2. Inhibition of autophagy attenuates endothelial hyperpermeability induced by LPS and LPS-conditioned medium. The relative endothelial permeability was assessed by a Transwell permeability assay after the indicated treatment. **(A)** HMEC-1 cells were treated with PBS (normal control, NC), LPS (5 μg/ml) alone, or LPS with autophagy inhibitors (NAC, 3-MA, CQ or BafA1) for 1 h ($n = 6$ per group). **(B)** HMEC-1 cells were transfected with luciferase shRNA (control; shLuc) or Atg5 shRNA (shAtg5). After selection with puromycin (250 ng/ml), the stable knockdown cell lines were treated with or without LPS (5 μg/ml) for 1 h ($n = 6$ per group). **(C)** Isolated human leukocytes were stimulated with PBS or 1 μg/ml LPS for 24 h, and the cell supernatants were collected as NC medium or LPS-conditioned medium. HUVECs were treated with LPS-conditioned medium or LPS (1 μg/ml) for 6 h. PMB (15 μg/ml) was used to rule out the possible effect of LPS activity on the LPS-conditioned medium ($n = 6$ per group). **(D)** HUVECs were co-treated with NC medium, LPS-conditioned medium, or LPS-conditioned medium and autophagy inhibitors (NAC, 3-MA, CQ or BafA1) for 6 h ($n = 6$ per group). ns: not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

i.p. injection of HCQ (65 mg/kg) 1.5 h after the *E. coli* infection). In addition, another set of experiments was designed to test the influence of drug administration at different times in mice. The mice were divided into the following four groups ($n = 6$ per group): Group 1 (control, intraperitoneal (i.p.) injection of PBS only), Group 2 (*E. coli* infection, i.p. injection of *E. coli* (5×10^9 CFU/kg) in PBS), Group 3 (pretreatment with HCQ, i.p. injection of HCQ (65 mg/kg) 1.5 h before the *E. coli* infection), and Group 4 (post-treatment with HCQ, i.p. injection of HCQ (65 mg/kg) 1.5 h after the *E. coli* infection). Blood was collected by orbital sinus sampling using heparinized capillary tubes at the indicated time point. The cytokine production and bacterial burden in the blood and the vascular leakage were determined to ensure the success of establishing bacterial sepsis in the mice. The survival rate of the mice was monitored every 6 h for the first 24 h after infection and every 12 h thereafter for up to 2 days, and the remaining mice were sacrificed by an overdose of anaesthetics.

Vascular leakage in the mouse organs

The *in vivo* endothelial permeability was measured using a modified Miles assay.[27] Briefly, 24 h after infection, the mice were intravenously injected with Evans blue dye (5%, 200 μl) 30 min before sacrifice. The organs (intestine and lung) from the mice were weighed and soaked in 0.5 ml of formamide (J.T. Baker, Phillipsburg, NJ) at 55 °C overnight to extract the Evans blue from the tissue. The amount of Evans blue in the extract was quantified by spectrometry at $OD_{600\,nm}$.

Vascular leakage in the mouse peritoneal cavity

Twenty-four hours after infection, the mice were intravenously injected with Evans blue dye (5%, 200 μl) 30 min before sacrifice. The peritoneal fluid was harvested from the mice by peritoneal washing with 2 ml PBS and centrifugation at $1000 \times g$ for 5 min. The $OD_{600\,nm}$ of the supernatants was determined, and the concentration of protein in the peritoneal washing fluid was determined using the Bradford method.

Determination of the bacterial CFU in blood and organs

Blood was collected from the orbital sinus of the mice by using heparinized capillary tubes at the indicated time points. The mice were sacrificed 24 h after infection. The organs (intestine, heart, and liver) of the mice were weighed and homogenized in 0.5 ml of PBS using a tissue homogenizer. Both the blood and the tissue homogenates were serially diluted. Thirty microlitres of each sample were plated on LB agar plates, and the bacterial colonies were enumerated after incubation at 37°C for 18 h.

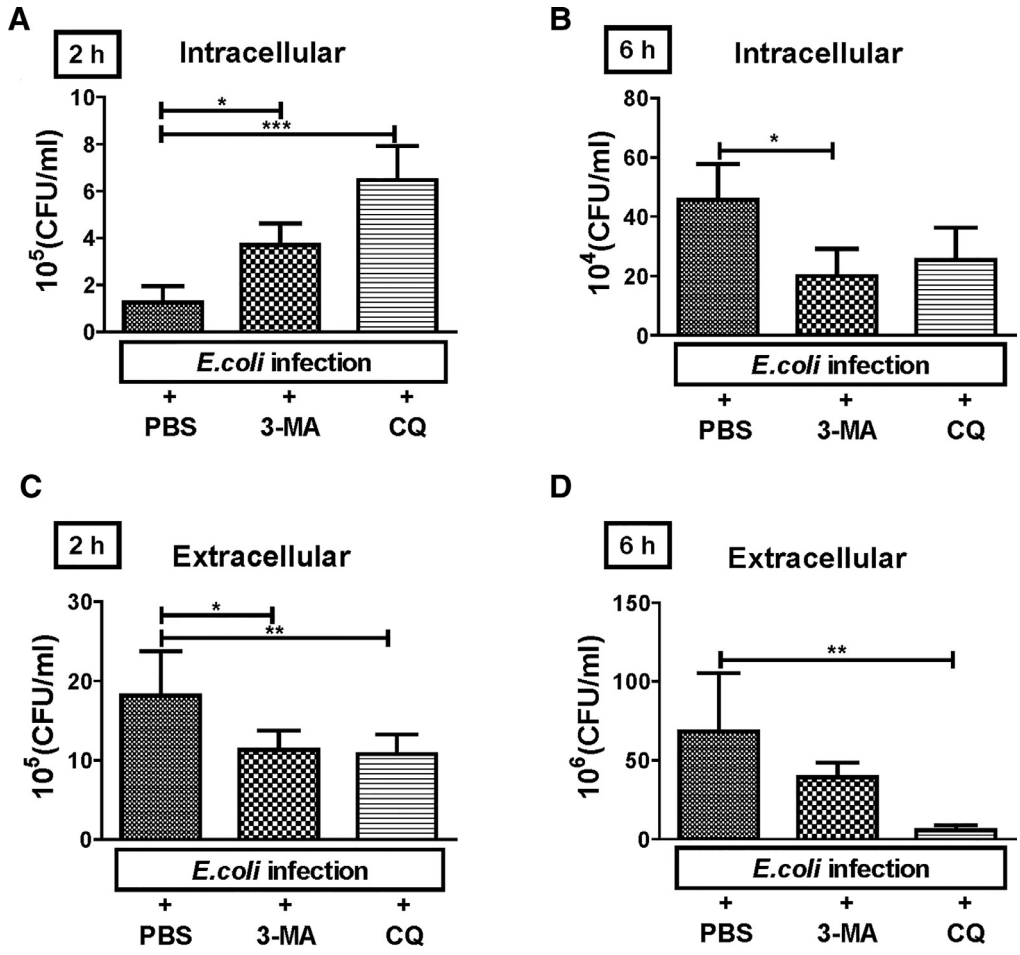

Fig. 3. Treatment with autophagy inhibitors enhances the phagocytosis of bacteria by human leucocytes. Leucocytes were infected with *E. coli* (MOI of 0.1) and co-treated with or without autophagy inhibitors (3-MA or CQ). The bacterial killing capacity was assessed by estimating the **(A)** intracellular and **(C)** extracellular CFUs 2 h after infection and the **(B)** intracellular and **(D)** extracellular CFUs 6 h after infection ($n = 4$ per group). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

Statistical analysis

The statistical analyses of each experiment were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test was used to compare the differences between two groups. For comparisons among multiple groups, the data were analysed by one-way analysis of variance (ANOVA). All data are presented as the means ± standard deviations (SDs). The differences between groups were considered statistically significant at $P<0.05$.

Results

LPS-induced cytokine secretion by human leucocytes is suppressed by the inhibition of autophagy

The roles of autophagy in a sepsis-induced cytokine storm were investigated by co-treating human leukocytes with LPS and autophagy inhibitors (3-MA or CQ). The results showed that LPS induced human leucocytes to secrete cytokines (IL-1β, IL-6, MIF and HMGB1), which could be supressed by the co-treatment with the autophagy inhibitors (Supplementary Fig. 1 and Fig. 1). The differences in the effects of LPS on cytokine secretion between a stable clone of LC3-knockdown THP-1 cells (shLC3) and a stable clone of vehicle control THP-1 cells (shGFP) were also compared. We found that the secretion of IL-6, MIF and HMGB1 was increased in the LPS-stimulated shGFP THP-1 cells and that this increase was significantly reduced in the LPS-stimulated shLC3 THP-1 cells (Supplementary Fig. 2). Taken together, these results suggest that autophagy is required for LPS-induced cytokine secretion by human leucocytes and that the inhibition of autophagy could attenuate the LPS-induced production of pro-inflammatory cytokines.

Endothelial hyperpermeability induced by either LPS or LPS-conditioned medium is attenuated in the presence of autophagy inhibitors

LPS and the leucocyte culture medium obtained after LPS stimulation (LPS-conditioned medium) were used as stimulators to induce hyperpermeability in the human endothelial cell line HMEC-1 and primary human umbilical vein endothelial cells (HUVECs). The cells were co-incubated with four different autophagy inhibitors (3-MA, NAC, BafA1, and CQ) that could block the autophagic process at different stages as indicated in the Materials and Methods and LPS or the LPS-conditioned medium. The results showed that LPS could induce endothelial cell hyperpermeability 1 h after stimulation; this effect was suppressed in the presence of the autophagy inhibitors (Fig. 2A). In addition, the knockdown of Atg5 (shAtg5) in the HMEC-1 cells prevented the LPS-induced increase in endothelial permeability observed in the shLuc HMEC-1 cells (Fig. 2B). Subsequently, we attempted to further confirm that the autophagy inhibitors could ameliorate cytokine-induced vascular leakage. HUVECs were incubated with LPS-conditioned medium collected 24 h after stimulating leucocytes with LPS to determine the effect of LPS-induced cytokines on endothelial permeability. As shown in Fig. 2C, the LPS-conditioned, but not NC medium

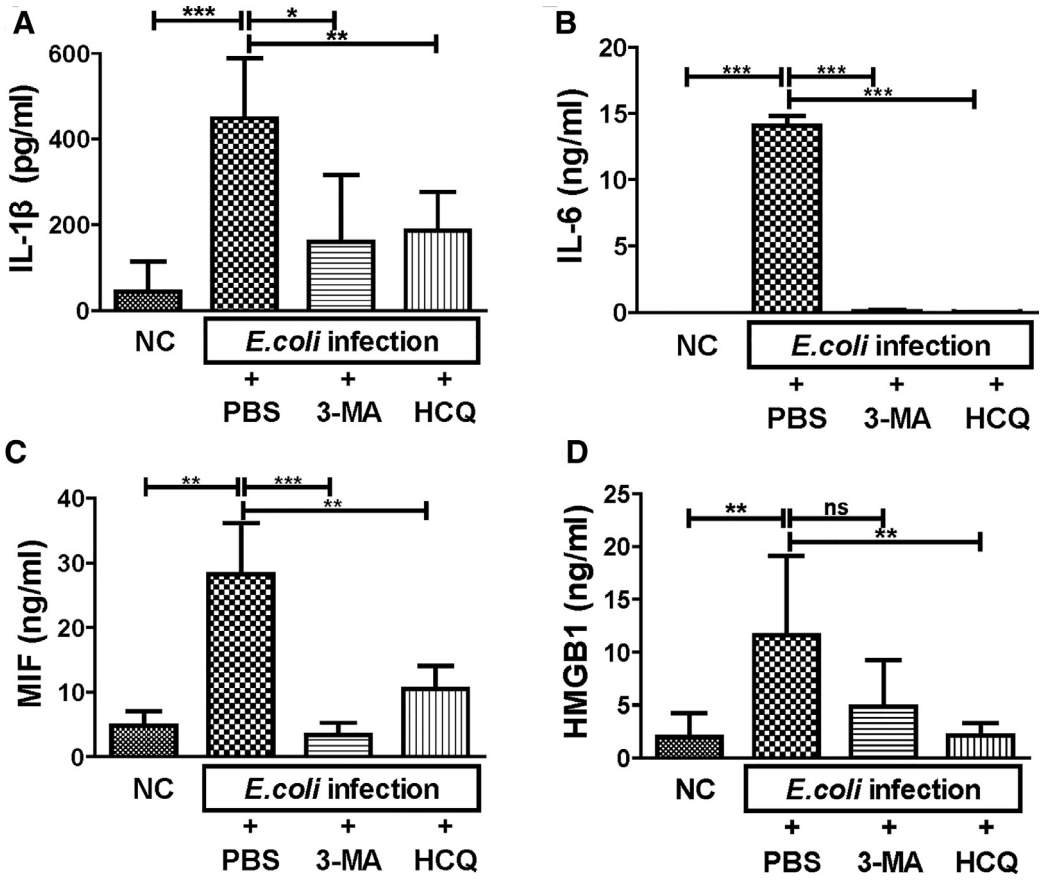

Fig. 4. Autophagy inhibitors inhibit cytokine secretion in *E. coli*-infected mice. BALB/c mice were intraperitoneally injected with PBS or *E. coli* (5×10^9 CFU/kg) and treated with or without autophagy inhibitors (40 mg/kg 3-MA or 65 mg/kg HCQ) 1.5 h after *E. coli* infection ($n = 6$ per group). Blood was collected, and the plasma level of **(A)** IL-1β, **(B)** IL-6, **(C)** MIF and **(D)** HMGB1 was analysed 24 h post-infection. ns: not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

significantly increased the permeability of HUVECs 6 h after incubation. Moreover, the hyperpermeability induced by the LPS-conditioned medium was not inhibited in the presence of PmB, and 1 μg/ml LPS could not increase HUVEC permeability. These results indicate that the hyperpermeability induced by the LPS-conditioned medium was caused by LPS-induced cytokines rather than by LPS. Furthermore, the hyperpermeability induced by the LPS-conditioned medium could be blocked in the presence of the autophagy inhibitors (Fig. 2D).

Treatment with autophagy inhibitors promotes bacterial clearance by leucocytes

Previous studies have indicated that macrophages deficient in autophagy exhibit increased uptake of bacteria.[23,24] In agreement with previous studies, compared to the PBS control group, we also found that the intracellular bacteria CFU was increased in the groups treated with the autophagy inhibitors (3-MA and CQ) at 2 h and decreased at 6 h after *E. coli* infection (Fig. 3A and 3B). In addition, the co-treatment with the autophagy inhibitors continuously decreased the extracellular bacteria CFU 2 and 6 h after the *E. coli* infection (Fig. 3C and 3D). These results suggest that autophagy inhibition could promote the uptake of *E. coli* without affecting the bacteria-killing ability of human leukocytes.

Autophagy inhibitors suppress cytokine secretion induced by E. coli infection in mice

The effect of autophagy inhibition on cytokine production *in vivo* was further evaluated in mice after *E. coli* infection. The results showed that infection by *E. coli* induced the production of pro-inflammatory cytokines, such as MIF, IL-6, IL-1β and HMGB1, in BALB/c mice in a time-dependent manner. The levels of these cytokines were significantly increased 24 h after infection (Supplementary Fig. 3). Furthermore, the treatment with the autophagy inhibitors (3-MA and HCQ) 1.5 h after infection effectively attenuated the *E. coli*-induced pro-inflammatory cytokine secretion (Fig. 4).

Autophagy inhibitors ameliorate vascular leakage in E. coli-infected mice

We also used an *in vivo* permeability assay and a peritoneal wash assay to confirm the protective effect of autophagy inhibitors against vascular leakage in *E. coli*-infected mice. The results showed that the treatment with the autophagy inhibitors (3-MA and HCQ) 1.5 h after infection could significantly reduce *E. coli*-induced vascular leakage in the lung, but not in the intestine, in BALB/c mice (Fig. 5A and 5B). In addition, the *E. coli*-induced increases in vascular permeability and protein extravasation in the peritoneal cavity were significantly blocked in the presence of the autophagy inhibitors (3-MA and HCQ) (Fig. 5C and 5D).

Post-treatment but not pretreatment with the autophagy inhibitor HCQ increases bacterial clearance and survival rate in E. coli-infected mice

As shown in Fig. 3, autophagy inhibitors might enhance bacterial clearance in leucocytes *in vitro*. We further investigated the influence of autophagy inhibition on the clearance of bacteria *in*

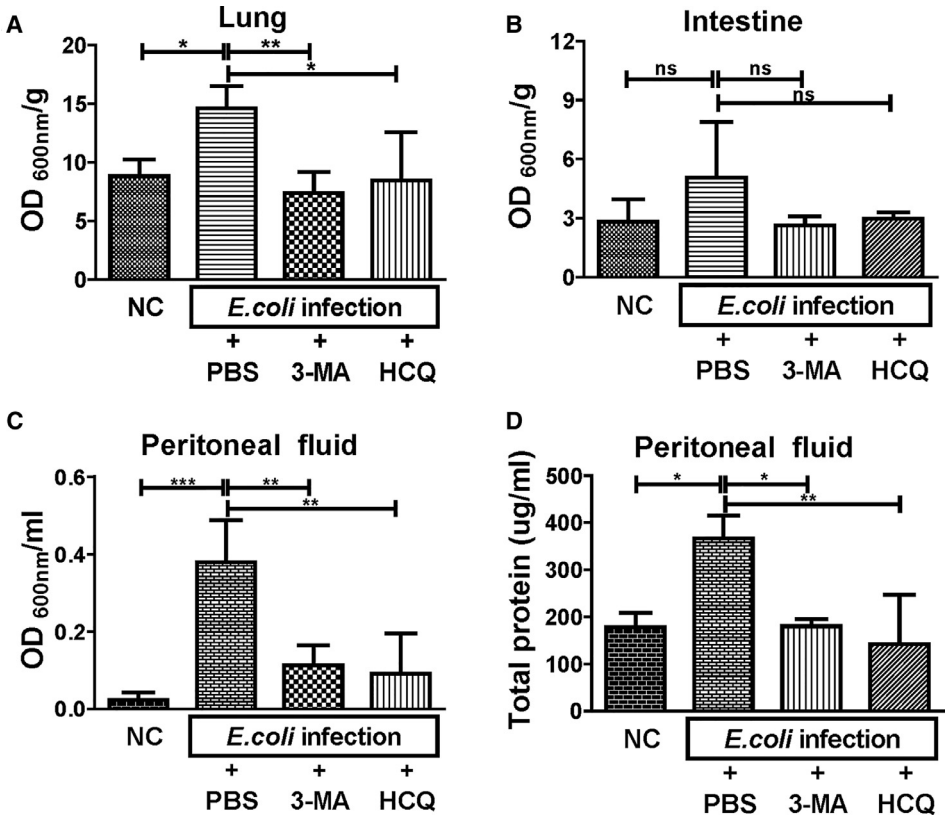

Fig. 5. Autophagy inhibitors prevent vascular leakage in *E. coli*-infected mice. BALB/c mice were intraperitoneally injected with PBS or *E. coli* (5×10^9 CFU/kg) and treated with or without autophagy inhibitors (40 mg/kg 3-MA or 65 mg/kg HCQ) 1.5 h after *E. coli* infection ($n = 6$ per group). Then, 5% Evans blue dye was intravenously injected into the mice's tail vein 30 min before sacrifice; the **(A)** lung, **(B)** intestine and **(C)** peritoneal washing fluid were collected; and the vascular permeability was detected by quantifying the extravasation of Evans blue dye using VersaMax microplate reader at OD $_{600\ nm}$. **(D)** The concentration of protein in the peritoneal washing fluid was quantified by the Bradford method. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

vivo. We compared the bacterial loads in different tissues from mice treated with or without the autophagy inhibitor HCQ 24 h after *E. coli* infection. The results showed that the mice treated with HCQ 1.5 h after *E. coli* infection (the post-treatment group) had a lower bacterial burden in the blood, intestine, heart, and liver than those in the non-treatment group (Fig. 6). However, the bacterial loads in the mice treated with HCQ 1.5 h before *E. coli* infection (the pretreatment group) were much higher than those in the non-treatment group 24 h post-infection (Fig. 6). Furthermore, the survival rate of the mice in the HCQ post-treatment group 48 h after *E. coli* infection was 100% (12/12), which was significantly higher than that in the non-treatment group (66%, 8/12). Conversely, the survival rate of the mice in the HCQ pretreatment group decreased to 37% (3/8) 48 h after *E. coli* infection (Fig. 7).

Discussion

In this study, we demonstrated that both LPS- and *E. coli*-induced cytokine production and vascular leakage were effectively attenuated by treatment with autophagy inhibitors both *in vitro* and *in vivo*. Furthermore, we also observed that treating mice with autophagy inhibitors after, but not before, *E. coli* infection could significantly decrease the bacteria load and increase the survival rate in mice. Therefore, the inhibition of autophagy may represent an alternative therapeutic strategy against sepsis.

Previous studies have shown that autophagy can regulate the secretion of cytokines that do not contain a secretory signal peptide, such as IL-1β and HMGB1, via an autophagosome/endosome-base secretion pathway.[16,29–32] Consistent with previous studies, we also demonstrated that the secretion of IL-1β and HMGB1 induced by either LPS or *E. coli* can be suppressed in the presence of autophagy inhibitors. In addition, we found that the secretion of IL-6, which contains a secretory signal peptide, and MIF, which is a cytokine that is secreted through a non-classical pathway involving an ABC-transporter, were both attenuated in the presence of the autophagy inhibitors.[28] Autophagy is known to enhance NF-κB signalling and regulate IL-6 and IL-8 secretion via a compartment intermixed with autophagic organelles.[29–31] Since the production of MIF is also regulated through NF-κB signalling,[32] it is possible that autophagy regulates MIF and IL-6 secretion/production through NF-κB activation. However, the precise mechanisms by which autophagy regulates the LPS- or *E. coli*-induced secretion of different cytokines require further investigation.

In our previous studies, we demonstrated that autophagy is involved in vascular leakage triggered by different stimulators, including MIF, dengue virus non-structural protein 1 and thrombin, via an autophagy-mediated adherent/junction protein degradation mechanism.[16,33] Previous studies also suggest that autophagy is involved in LPS-induced endothelial hyperpermeability.[13,34] In this study, we further demonstrated that the vascular leakage induced by LPS, LPS-conditioned medium, and *E. coli* infection was also attenuated by autophagy inhibitors. Therefore, autophagy may represent a common pathway involved in endothelial hyperpermeability during infection or inflammation.

In addition to the suppression of LPS- or *E. coli*-induced cytokine secretion and vascular leakage, surprisingly, the bacterial clearance by leucocytes was found to be enhanced in the presence of the autophagy inhibitors. Traditionally, autophagy is considered a lysosomal degradation pathway that plays key roles in bacterial clearance.[35] During infection, intracellular pathogens

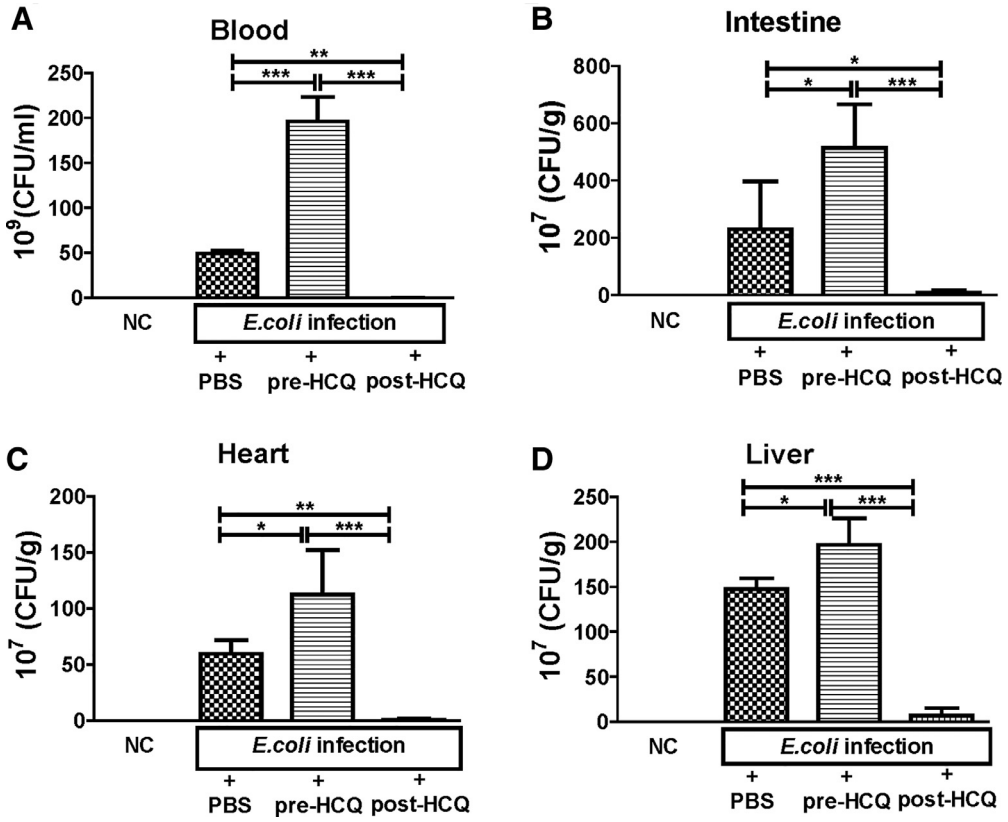

Fig. 6. Post-treatment but not pretreatment with the autophagy inhibitor HCQ enhances bacterial clearance in *E. coli*-infected mice. HCQ (65 mg/kg) was intraperitoneally injected into BALB/c mice 1.5 h before or after *E. coli* (5×10^9 CFU/kg) intraperitoneal infection ($n = 6$ per group). At 24 h post-infection, the mice were sacrificed, and the bacterial burden in the **(A)** blood, **(B)** intestine, **(C)** heart, or **(D)** liver was analysed by plating the homogenates on LB agar.

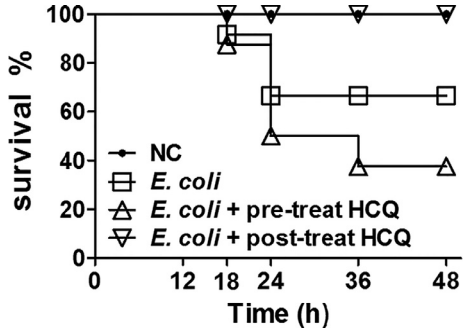

Fig. 7. Post-treatment but not pretreatment with the autophagy inhibitor HCQ protects mice against lethal *E. coli* infection. HCQ (65 mg/kg) was intraperitoneally injected into BALB/c mice ($n = 7$-10 per group) 1.5 h before or after intraperitoneal infection with *E. coli* (5×10^9 CFU/kg). The survival rate of the mice was monitored at the indicated time points after infection. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

are recognized and ubiquitinated for recruitment to autophagic cargo receptors; this process results in the transportation of these pathogens to autophagosomes, which eventually fuse with lysosomes to kill the pathogens.[36,37] However, the roles of autophagy in immune cells to engulf bacteria are still controversial.[38] Intriguingly, macrophages from Atg7 knockout mice exhibit an increase in the expression of scavenger receptors and higher bacterial uptake than those from wild type mice in response to mycobacterial infection.[39] In addition, the inhibition of autophagy can increase the phagocytosis of macrophages to methicillin-resistant staphylococcus aureus.[24] However, the phagocytosis of pneumococci by neutrophils is enhanced by autophagy.[40] Therefore, the effect of autophagy inhibitors on the phagocytosis and bactericidal activity of different immune cells against different bacteria should be further studied.

Interestingly, in this study, we found that only post-treatment with HCQ could effectively inhibit bacterial infection and increase survival in mice. However, the mice pre-treated with HCQ showed significantly higher tissue bacterial loads and higher mortality rates than the non-treated mice. These data suggest that autophagy is a double-edged sword in sepsis. During the early stages of infection, autophagy is required for pro-inflammatory cytokine secretion to trigger a beneficial inflammatory response to remove pathogens. However, the overwhelming production of these cytokines and autophagy-mediated vascular leakage can lead to multiple organ failure and shock during sepsis, which may explain why the differently timed treatments with the autophagy inhibitors had completely different outcomes in the septic mice. The identification of the therapeutic window during which treatment with autophagy inhibitors can stop the inflammatory cascade in a timely manner should be pursued in the future.

Taken together, our results indicate that inhibition of autophagy at either initiation stage by 3-MA or autophagosome and lysosome fusion stage by CQ after *E. coli* infection have several beneficial effects. First, the clearance of bacteria by immune cells can be enhanced in the presence of autophagy inhibitors, which can prevent the spread of local infection throughout the body. Second mitochondrial function may be maintained in cells due to the blockage of mitophagy by autophagy inhibitors, and ROS generated by mitochondria can be further used to eliminate intracellular pathogens.[41,42] Finally, importantly, autophagy inhibitors can inhibit *E. coli*-induced cytokine secretion, thus preventing the overproduction of pro-inflammatory cytokines and the development of endothelial hyperpermeability in mice. Taken together, these findings may explain why the post-treatment of mice with HCQ could

protect against *E. coli*-induced lethality. Since HCQ is a recognized antimalarial and anti-inflammatory drug, its safety and tolerability are well documented.[43] Clinical trials investigating HCQ should be performed in the future to validate its efficacy in improving the therapeutic outcome in septic patients.

Conflict of interest

The authors have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2018.12.003.

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