Simvastatin inhibits the inflammation and oxidative stress of human neutrophils in sepsis via autophagy induction

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Abstract. Simvastatin exerts a protective effect during sepsis (SP) in animal models; however, the underlying mechanism is not completely understood, particularly in human SP. Neutrophils are a critical effector in the host inflammatory response to SP. Therefore, the present study aimed to investigate the effect of simvastatin on neutrophils in human SP. Neutrophils were isolated from the peripheral venous blood of adult patients with SP and healthy volunteers (HP). Cell viability was analyzed using the MTT assay. Intracellular reactive oxygen species (ROS) generation and the concentrations of inflammatory mediators were also assessed using flow cytometry and ELISA. The results demonstrated that the cell viability of neutrophils from the SP group was significantly decreased compared with that in the HP group, and that treatment with simvastatin partly reversed the reduced cell viability. Furthermore, simvastatin administration significantly decreased ROS production and the concentrations of TNF-α and IL-6, which were significantly increased in neutrophils isolated from the SP group. Simvastatin also enhanced autophagy induction, as indicated by the promotion of the conversion of LC3I to LC3II and the increased expression levels of Beclin 1 in SP neutrophils. Treatment with 3-methyladenine, an autophagy inhibitor, completely blocked the protective effects of simvastatin on neutrophils from SP, including the effects of simvastatin on the inhibition of inflammation, oxidative stress and improving cell viability. Collectively, the present study provided evidence for the simvastatin-induced autophagic process of neutrophils involved in human SP, which protects neutrophils and partially attenuates the inflammatory response and oxidative stress. Therefore, the augmentation of neutrophil autophagy may serve as a potential therapeutic target for patients with SP.

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Introduction

Sepsis (SP), a complex clinical condition, is characterized by a systemic inflammatory response that results in tissue or organ hypoperfusion and hypoxia, as well as organ dysfunction (1). Despite significant efforts in the development of therapeutic strategies, SP remains life-threatening and is one of the most common causes of mortality (15.9% in United States) in intensive care units (ICUs) (1-3). Recent studies focusing on the pathophysiology of SP have reported that systemic inflammation leads to disorders of inflammation/anti-inflammation and oxidant/antioxidant processes, the increased production of adhesion molecules and chemokines, and the dysregulation of apoptotic cell death (4,5). It is considered that neutrophils serve an important role in the host response to infection and SP, and are recruited in large numbers to the site of damage tissue (6).

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are a widely used medication for controlling hypercholesterolemia via a lipid-lowering effect (7). Recently, numerous studies have reported that statins possess a variety of other biological functions, including regulation of the inflammatory response, exerting immunomodulatory effects and protecting against cell apoptosis in animal models of SP (8,9). Autophagy, a well-conserved homeostatic cellular system responsible for removing damaged or dysfunctional cellular organelles, is essential for the protection of cell survival against stressors, such as invasive pathogenic organisms and reactive oxygen species (ROS) (10). Previous studies have shown that the regulatory functions of autophagy may contribute to SP-related organ dysfunction (11,12). Moreover, several recent reports have identified the relationship between autophagy activity and simvastatin (13,14). Based on the aforementioned evidence, the present study aimed to investigate the impact of simvastatin on inflammation and ROS regulation, as well as the association of these processes with simvastatin-induced autophagy in neutrophils from patients with SP.

Materials and methods

Reagents and antibodies. Simvastatin (Sigma-Aldrich; Merck KGaA) was dissolved in DMSO to make a 50 mM stock solution, which was maintained at 4°C before use. The MTT and

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) assays were purchased from Sigma-Aldrich (Merck KGaA) and Nanjing KeyGen Biotech Co., Ltd., respectively. Antibodies targeted against Beclin 1, LC3 and GAPDH were purchased from Abcam. RPMI-1640 and FBS were purchased from Gibco (Thermo Fisher Scientific, Inc.). ELISA kits were purchased from Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.

Human neutrophil isolation. Peripheral venous blood samples (10 ml) were obtained from six healthy volunteers (HP) and six patients with gram-negative SP, who were admitted to the ICU of the Department of Anesthesiology of The First Affiliated Hospital of Soochow University (Suzhou, China) between January 2019 and December 2019. All study participants or their relatives (age, ≥18 years) provided written informed consent in accordance with the Declaration of Helsinki. The present study was approved by the Institutional Review Board and Ethics committee of The First Affiliated Hospital of Soochow University (approval no. 138/2018). The inclusion criteria were as follows: i) Age, ≥18 years; and ii) diagnosed with SP based on the third international consensus definitions for sepsis and septic shock (Sepsis-3) (15). Furthermore, the exclusion criteria were as follows: i) HIV infection; ii) organ transplantation; and iii) pregnancy. The mean age of the participants was 55.0±10.9 years for the SP group and 57.8±10.5 years for the HP group. There were three males and three females in both the SP and HP groups. Venous blood samples were collected within 1 h after enrollment. Human neutrophils were isolated by discontinuous density gradient centrifugation as previously described (16). Briefly, the leukocyte-enriched pellets were collected by centrifugation at 250 x g for 6 min at room temperature. Subsequently, in a centrifuge tube, 3 ml 65% Percoll solution (Pharmacia Biotech) was layered onto 3 ml 75% Percoll solution, followed by the layering of 4 ml of peripheral blood. The tube was then centrifuged at 330 x g for 25 min at room temperature and the interface between the 75 and 65% layers was carefully aspirated. After hypotonic lysis of erythrocytes by RBC Lysis Buffer (Roche Diagnostics), neutrophils were isolated to reach 98% purity as determined by Giemsa staining. After isolation, 1x106 cells were fixed in methanol for 5 min at room temperature and stained with Giemsa solution for 1 min at room temperature and were subsequently imaged using light microscopy. The viability of neutrophils was >95% as indicated by Trypan blue staining. Briefly, neutrophil suspensions were added to an equal volume of 0.4% trypan blue solution in a microtube and mixed for 2 min on ice. Neutrophil viability was analyzed using a Neubauer chamber and an optic microscope.

Cell culture and stimulation. Neutrophils were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C. Cells were adjusted to ~$1x10^6$ cells/ml in RPMI-l640 medium supplemented with 10% FBS and 2 mM L-glutamine, and then plated in 96-well culture plates. Cells were treated with vehicle (DMSO) or simvastatin (5 μM) for 24 h. For inhibitory assays, cells were pre-incubated with the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium (DPI; 20 μM) or the autophagy inhibitor 3-methyladenine (3-MA; 5 nM) for 1 h at 37˚C before each experiment as previously described (17,18). 3-MA and DPI were both dissolved in DMSO and diluted to the working concentrations. DMSO was used as the negative control. Neutrophil media were centrifuged at 400 x g for 5 min at 4˚C, and the supernatants were used for quantification of cytokines (TNF-α and IL-6).

Cell viability assay. Cell viability was detected using the MTT assay. After treatment for 24 h, MTT solution was added to the medium and incubated at 37˚C for 4 h. Subsequently, the MTT solution was replaced with DMSO (200 μl) for 30 min at 37˚C. The optical density was assessed using a microplate reader (Bio-Rad Laboratories, Inc.). Neutrophil viability was analyzed at different doses of SIM (0, 1, 5, 10 or 15 μM) for 24 h to assess the lethal concentration of SIM. The mean absorbance values were recorded and normalized to the values obtained for cells cultured with vehicle.

Measurement of intracellular ROS levels. For intracellular ROS assessment, cells were seeded into plates ($1x10^5$ cells/well). After treatment, cells were incubated with DCFH-DA (50 μM; Nanjing KeyGen Biotech Co., Ltd.) at 37˚C for 20 min. DCF fluorescence was determined and analyzed using a flow cytometer (FACSVerse; BD Biosciences) and FlowJo version 10 (Tree Star, Inc.).

Western blotting. Cells were lysed with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA and protease inhibitor cocktail). After centrifugation at 12,000 x g for 10 min at 4˚C, the supernatants were collected. For western blotting, 30 μg of protein/lane was separated by SDS-PAGE on a 15% gel and then transferred to a PVDF membrane. After blocking with 5% non-fat milk in TBST (10 mM Tris, 150 mM NaCl and 0.1% Tween-20) for 1 h at 37˚C, the membranes were probed with primary antibodies targeted against rabbit anti-Beclin-1 (1:2,000; cat. no. ab210498; Abcam), rabbit anti-LC3 (1:1,000; cat. no. ab192890; Abcam) or rabbit anti-GAPDH (1:1,000; cat. no. ab181602; Abcam) with gentle agitation overnight at 4˚C. After washing with TBST, the membranes were further incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:5,000; cat. no. ab205718; Abcam) in blocking buffer for 1 h at room temperature. Specific protein bands were visualized using the ECL detection kit (Amersham; Cytiva). The blots were scanned, and the intensity of the obtained blot bands was semi-quantified and analyzed using Science Lab Image Gauge software (version 4.0; FUJIFILM Wako Pure Chemical Corporation).

Quantification of cytokines. The concentrations of TNF-α and IL-6 in the supernatants were determined using Human TNF-α Quantikine ELISA Kit (cat. no. DTA00D; R&D Systems, Inc.) and Human IL-6 Quantikine ELISA Kit (cat. no. D6050; R&D Systems, Inc.) according to the manufacturer's protocols. Absorbance was determined at a wavelength of 450 nm using a VersaMax microplate reader (Molecular Devices, LLC). The concentrations were calculated according to calibration curves.

Statistical analysis. Statistical analyses were performed using SPSS software (version 22.0; IBM Corp.). Data are presented

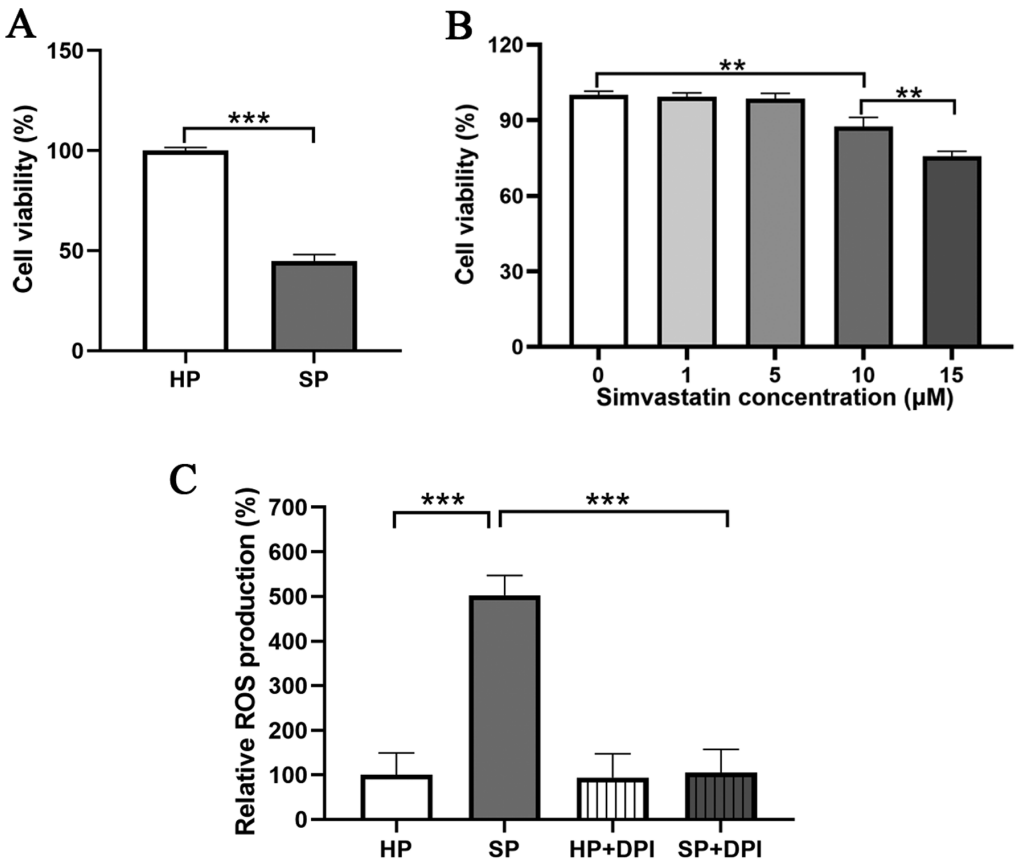

Figure 1. Cell viability and ROS production of neutrophils. (A) Cell viability of neutrophils was significantly decreased in the SP group compared with that in the HP group. (B) At 24 h post-treatment, the dose-dependent effects of simvastatin on the cell viability of neutrophils were observed. (C) A significantly higher production of ROS was observed in neutrophils from the SP group compared with those from the HP group. The increased production of ROS by neutrophils from the SP group was significantly reversed by treatment with DPI. **P<0.01 and ***P<0.001. ROS, reactive oxygen species; SP, sepsis; HP, healthy volunteers; DPI, diphenyleneiodonium.

as the mean ± SD from three replicates. Comparisons among multiple groups were analyzed using one-way or two-way ANOVA followed by Bonferroni's post hoc test. The unpaired Student's t-test was used for comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Simvastatin protects neutrophils in SP. To investigate the viability of neutrophils in SP, an MTT assay was performed. The results demonstrated that the viability of neutrophils was significantly decreased in the SP group compared with that in the HP group (Fig. 1A).

Next, whether simvastatin could protect neutrophils from SP was investigated. To obtain a non-toxic dose of the simvastatin, a viability test was performed, in which the neutrophils from the HP group were treated with different doses of simvastatin (0, 1, 5, 10 or 15 μM) for 24 h to assess the lethal concentration. A significant decrease in neutrophil viability was observed following treatment with 10 or 15 μM simvastatin compared with that in untreated neutrophils (Fig. 1B). Moreover, there was no significant difference in viability between the 0 and 5 μM simvastatin groups, thus 5 μM was considered as the optimal concentration in the present study. Subsequently, neutrophils from the SP and HP groups were treated with simvastatin (5 μM), and the results indicated that the viability of neutrophils was 98.33% in the HP group, but 42.00% in the SP group (Fig. 2A). Moreover, simvastatin significantly inhibited the decreased viability of neutrophils in the SP group (42.00 vs. 71.33%, P<0.01).

Simvastatin decreases intracellular ROS levels in neutrophils. Subsequently, ROS production in neutrophils was assessed. The results demonstrated that the neutrophils from the SP group displayed a significantly higher production of ROS compared with neutrophils from the HP group. In addition, the elevated level of ROS was abrogated with treatment by DPI, an inhibitor of NADPH oxidase (Figs. 1C and S1). Following treatment with simvastatin, neutrophils in the SP group displayed significantly decreased levels of ROS (223.33±30.86%) compared with those from the untreated SP group (606.67±38.24%, P<0.001; Fig. 2B). Furthermore, simvastatin did not affect ROS production in neutrophils from the HP group.

Simvastatin reduces inflammation in SP. To investigate whether simvastatin had an effect on the inflammatory response in neutrophils, the concentrations of TNF-α and IL-6 in the supernatants of neutrophil-conditioned media were assessed. The results demonstrated that the concentrations of TNF-α and IL-6 were significantly higher in the SP

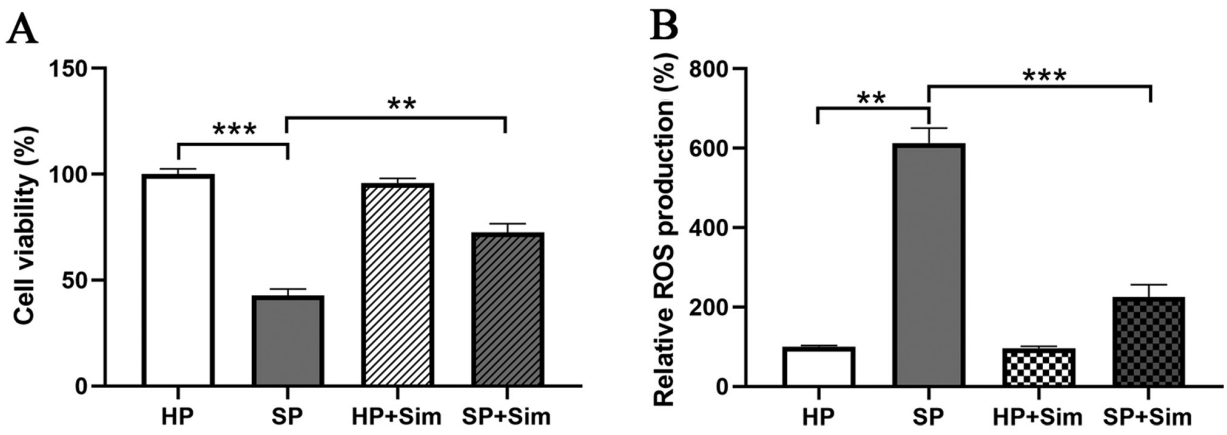

Figure 2. Sim improves cell viability and decreases intracellular ROS levels in neutrophils from the SP group. (A) Treatment with Sim partly reversed the inhibition of cell viability of neutrophils in the SP group. (B) A significantly higher production of ROS was observed in neutrophils from the SP group compared with those from the HP group. Sim significantly decreased ROS production in neutrophils from the SP group. **P<0.01 and ***P<0.001. Sim, simvastatin; ROS, reactive oxygen species; SP, sepsis; HP, healthy volunteers.

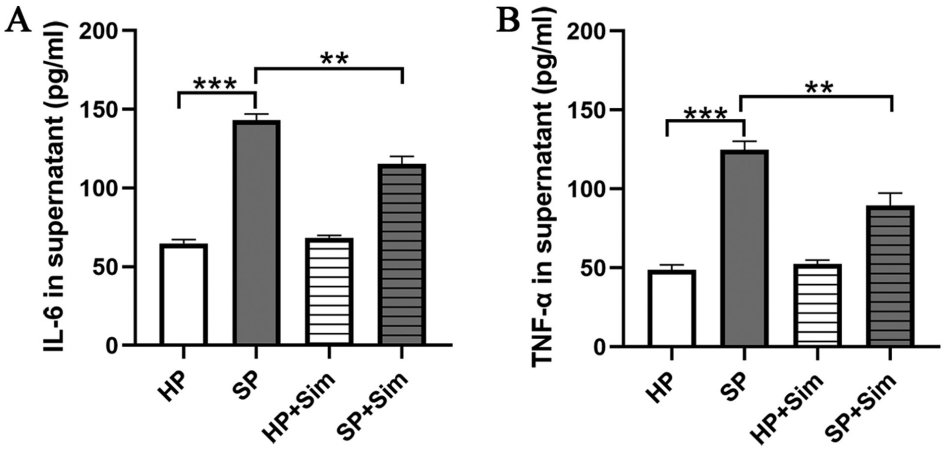

Figure 3. Sim decreases the concentrations of proinflammatory cytokines in neutrophil-conditioned media from the SP group. Concentrations of (A) IL-6 and (B) TNF-α were significantly higher in neutrophil-conditioned media of the SP group compared with those in the HP group. IL-6 and TNF-α concentrations were significantly reduced after treatment with Sim in the SP group. **P<0.01 and ***P<0.001. Sim, simvastatin; SP, sepsis; HP, healthy volunteers.

neutrophil-conditioned media compared with those in the HP neutrophil-conditioned media (Fig. 3A and B). However, TNF-α and IL-6 concentrations were significantly reduced after treatment with simvastatin in septic neutrophils (SP vs. SP + Sim TNF-α, 124.67±5.51 vs. 89.33±8.14 pg/ml, P=0.003; SP vs. SP + Sim, IL-6, 142.67±4.04 vs. 115.33±4.73 pg/ml, P=0.002).

Simvastatin inhibits inflammation and oxidative stress via autophagy induction in SP. Subsequently, whether simvastatin affected autophagy in septic neutrophils was investigated. The conversion of LC3I to LC3II was significantly increased in neutrophils from the SP group compared with that in the HP group (Fig. 4A). Moreover, SP neutrophils exhibited a significant increase in Beclin 1 protein expression (another autophagy-related molecule) compared with HP neutrophils (P<0.001; Fig. 4B). Notably, treatment with simvastatin promoted the conversion of LC3I to LC3II and elevated the expression level of Beclin1 in SP neutrophils compared with the untreated SP group, and these effects were abrogated by treatment with 3-MA, a well-known autophagy inhibitor (Fig. 4C and D). These results indicated that simvastatin enhanced autophagy in neutrophils from the SP group.

To investigate the importance of autophagy activation in the protective effect of simvastatin, neutrophils were treated with 3-MA. The results demonstrated that 3-MA administration completely blocked the protective effect of simvastatin, as evidenced by the decreased viability of neutrophils compared with that in the SP group treated with simvastatin alone (SP + Sim vs. SP + Sim + 3-MA, 72.33±6.03 vs. 41.67±3.51%, P=0.002; Fig. 5A). Furthermore, the inhibition of ROS production by simvastatin was reversed by treatment with 3-MA in septic neutrophils (SP + Sim vs. SP + Sim + 3-MA, 217.67±18.23 vs. 566.67±31.34, P<0.001; Fig. 5B). Subsequently, the role of autophagy in the inflammatory regulation of simvastatin was examined. It was found that the suppression of autophagy using 3-MA eliminated the inflammatory inhibitory effect of simvastatin in septic neutrophils, as evidenced by the increased concentrations of IL-6 (SP + Sim vs. SP + Sim + 3-MA, 109.00±6.00 vs. 143.67±5.69 pg/ml, P=0.002) and TNF-α (SP + Sim vs. SP + Sim + 3-MA, 81.67±3.06 vs. 114.00±6.24 pg/ml, P=0.001; Fig. 5C and D).

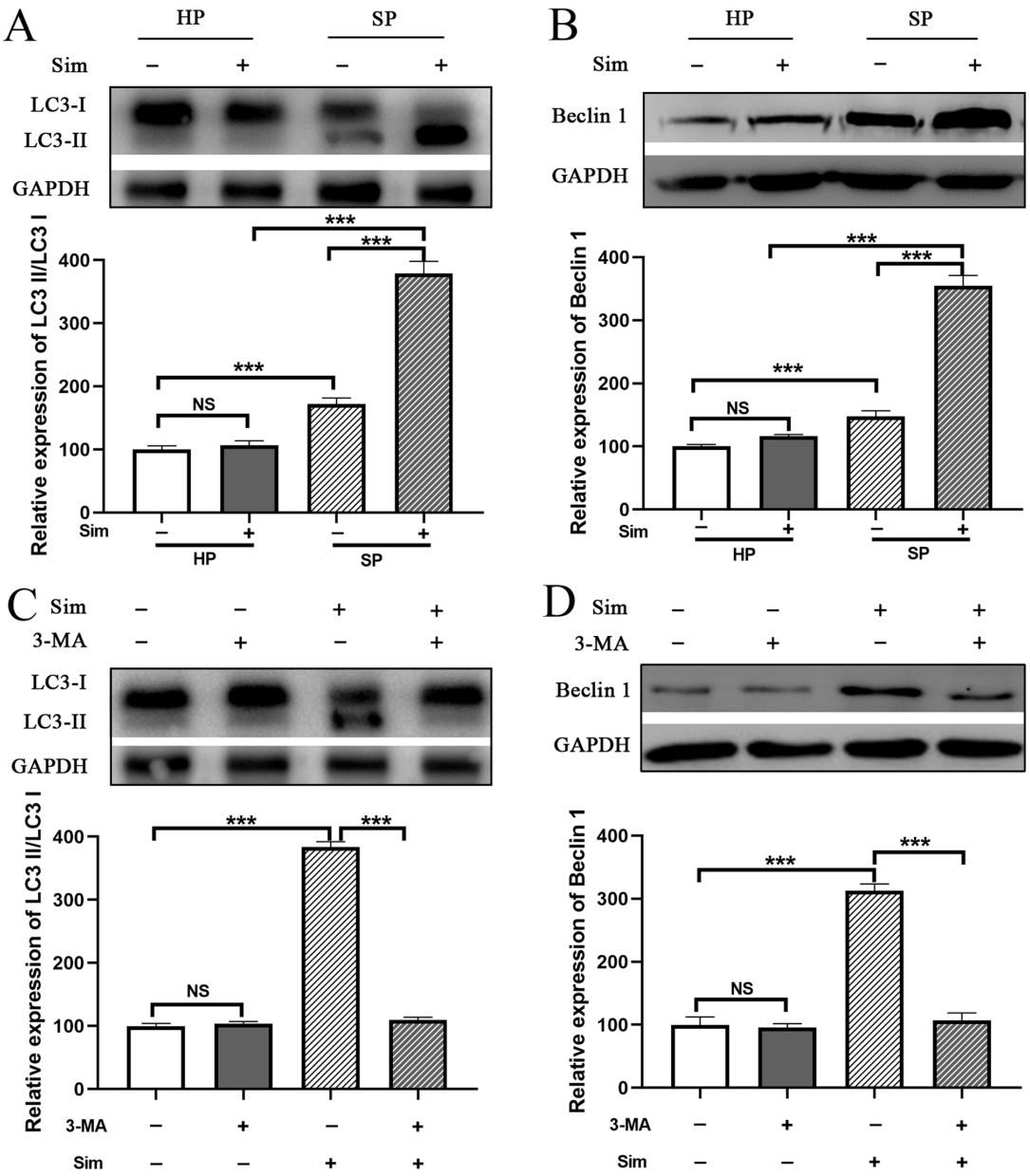

Figure 4. Sim enhances autophagy induction in neutrophils. (A) LC3I to LC3II conversion and (B) the expression levels of Beclin 1 were significantly increased in neutrophils from the SP group compared with those in the HP group. Treatment with Sim significantly promoted the conversion of LC3I to LC3II and elevated the expression level of Beclin 1 in SP neutrophils. Treatment with 3-MA significantly reversed Sim-induced (C) conversion of LC3I to LC3II and (D) the expression levels of Beclin 1 in neutrophils from the SP group. ***P<0.001. Sim, simvastatin; SP, sepsis; HP, healthy volunteers; 3-MA, 3-methyladenine; NS, not significant.

Discussion

SP is a life-threatening organ dysfunction disease caused by a dysregulated host response to infection, with a mortality rate of 28.6% in patients with severe SP in the United States (19). Neutrophils, the most abundant inflammatory cells in the circulating bloodstream, serve an important role in the SP, which phagocytose and eradicate invasive substances via phagocytosis, degranulation, the release of inflammatory cytokines and the generation of neutrophil extracellular traps (8,9,20). Numerous studies demonstrated a protective effect of simvastatin against SP in an animal model of abdominal SP and the majority of studies focusing on the association between statins and SP have been conducted in experimental animals (21,22). In the present study, simvastatin treatment led to the protection of neutrophils, decreased proinflammatory cytokine concentrations and attenuated oxidative stress in human neutrophils from patients with SP via autophagy enhancement.

The excessive production of ROS causes significant cellular dysfunctions in organs, which may also contribute to multi-organ system failures (23). High levels of ROS promoted apoptosis by opening mitochondrial permeability transition pores during experimental SP (24), whereas inhibition of ROS generation exhibited a protective effect in mice with SP (25,26). In the present study, the levels of ROS were elevated in human septic neutrophils. Moreover, treatment with DPI, an NADPH oxidase inhibitor, reversed the high generation of ROS in human septic neutrophils, indicating the sources of ROS in

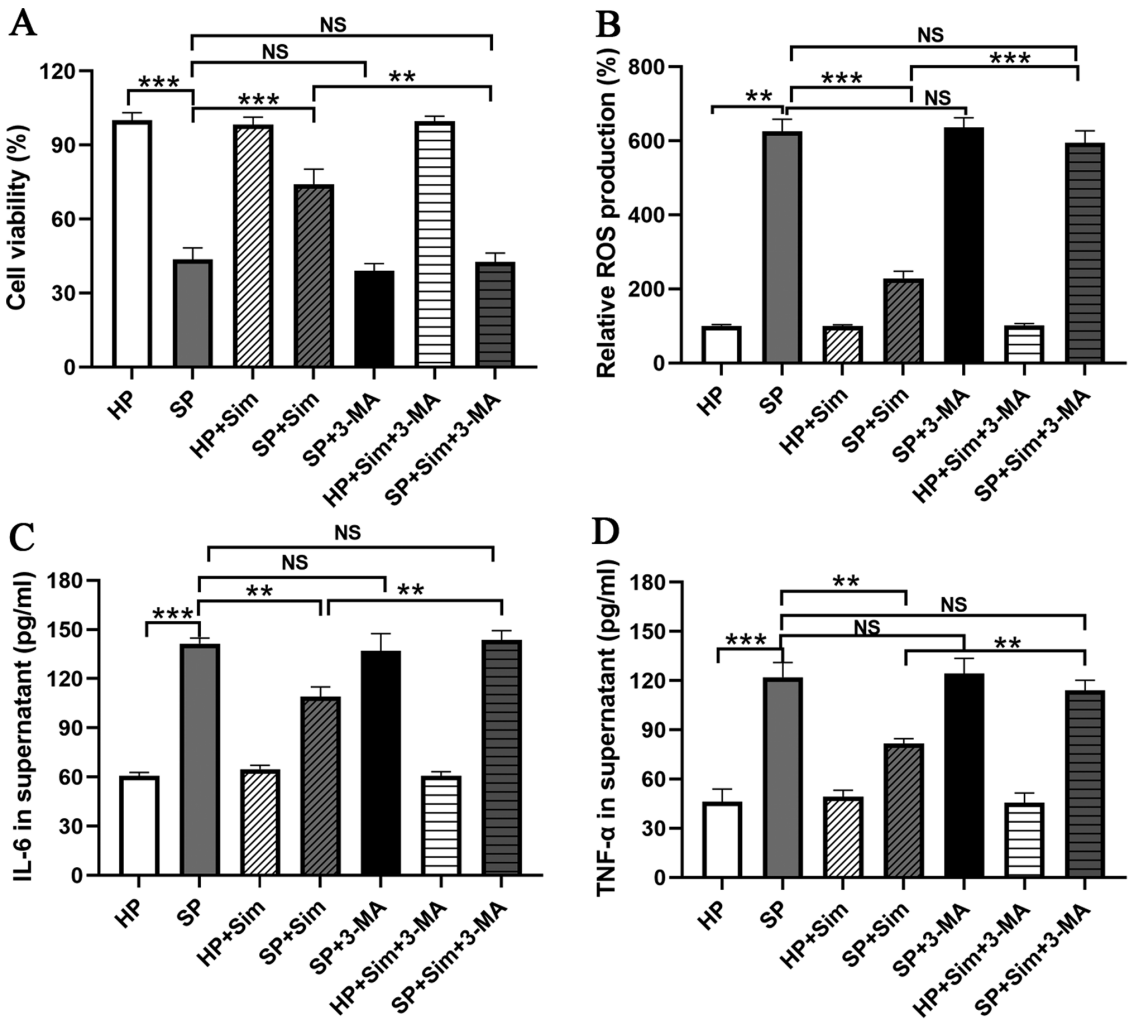

Figure 5. Sim inhibits inflammation and oxidative stress via autophagy induction. Treatment with 3-MA, an autophagy inhibitor, blocked the protective effect of Sim on neutrophils in the SP group, including (A) decreasing the cell viability of neutrophils, (B) increasing ROS production and increasing the concentrations of (C) IL-6 and (D) TNF-α in neutrophil-conditioned media. **P<0.01 and ***P<0.001. Sim, simvastatin; 3-MA, 3-methyladenine; SP, sepsis; ROS, reactive oxygen species; HP, healthy volunteers; NS, not significant;

human septic neutrophils primarily rely on NADPH oxidase. NADPH oxidase is an important enzyme-rich cytoplasmic granule contained in neutrophils that can convert molecular oxygen into free radicals of oxygen, superoxide anions and H_2O_2 (27). In addition, treatment with simvastatin significantly attenuated ROS levels in neutrophils isolated from patients with SP, which was consistent with that of a previous study that reported that simvastatin administration reduced brain oxidative stress in experimental SP (28).

Furthermore, the role of simvastatin in proinflammatory cytokine expression in SP neutrophils was investigated. Cytokines have been shown to serve critical roles in SP via the regulation of the immune response to infection (29,30). The present study observed that simvastatin decreased the concentrations of IL-6 and TNF-α in human septic neutrophils. IL-6 and TNF-α are two major proinflammatory cytokines that stimulate systematic inflammation. These act as endogenous pyrogens, upregulating the generation of other proinflammatory cytokines, as well as promoting the generation of acute phase proteins (31). A previous study reported that IL-6 was the key cytokine in the pathogenesis of severe SP and an increased level of IL-6 was associated with a higher mortality rate in patients with SP (32). Another proinflammatory cytokine, TNF-α, has also been revealed to be significantly increased in patients who developed and died of SP (33). In addition, use of a soluble TNF-α surface receptor blocked cytokine functions, and subsequently reduced the morbidity and mortality associated with septic shock in animal models (34).

The extent of immune cell death is strongly associated with the severity and mortality of SP; however, the pathological role of immune cell death in SP is not completely understood (35). The following mechanisms are considered to be important, including damage-associated molecular patterns, histone release, neutrophil extracellular traps and autophagy (36,37). Autophagy is an essential intracellular degradation process that is critical for cell survival and homoeostasis maintenance, which has been shown to serve a crucial role for autophagy in the regulation of SP (38,39). In the present study, it was found that autophagy in neutrophils from patients with SP served an important role in maintaining the innate effector functions of neutrophils. Neutrophils isolated from the SP group exhibited elevated autophagy induction. Moreover, simvastatin intervention enhanced autophagy activity in neutrophils isolated from patients with SP via the elevated expression

of Beclin 1 and the increased conversion of LC3I to LC3II. Bruiners *et al* (13) demonstrated that simvastatin treatment of human cells had transcriptional effects on mechanistic target of rapamycin complex 1 and AMP-activated protein kinase signaling pathways, thereby promoting autophagy. Moreover, lysosome-associated membrane glycoprotein-1 and Unc-51 like autophagy activating kinase 1 have been shown to be involved in simvastatin-induced autophagy (40). Recently, the mechanism of action underlying autophagy in SP has received increased attention. Autophagy has been revealed as a cellular adaptive protective mechanism in SP, where it could eliminate damaged proteins, bacteria and pathogens in the cytoplasm (39). Herein, the present study also demonstrated that inhibition of autophagy using 3-MA reversed the effects of simvastatin on cell viability, the production of ROS and the release of proinflammatory mediators (TNF-α and IL-6) in septic neutrophils. These findings were consistent with previous studies that reported that the autophagy regulation mechanism in SP was involved in oxidative stress, immune regulation and inflammation regulation in animal models (41,42).

In conclusion, the present study provided novel evidence for the simvastatin-induced autophagic process of neutrophils involved in human SP, which subsequently attenuated the inflammatory response and oxidative stress. These results suggested that autophagy served a crucial role in the protective effect of simvastatin in human SP and that augmentation of neutrophil autophagy may be a potential therapeutic target for patients with SP. Moreover, the relationship between simvastatin and autophagy in human septic neutrophils should be investigated further in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available on reasonable request from the corresponding author.

Authors' contributions

LX and XW performed the cell experiments. XW performed the MTT and flow cytometry assays. YKZ and MH performed the western blot assays. LX and MH performed the ELISAs. LX and JC contributed to the study design and writing of the manuscript. LX and JC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board and Ethics committee of The First Affiliated Hospital of Soochow University (Suzhou, China; approval no. 138/2018).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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