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Original Article

Ephedrine hydrochloride protects mice from *staphylococcus aureus*-induced peritonitis

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Abstract: *Staphylococcus aureus* is a Gram-positive (G^+) bacterium that causes a wide range of diseases in humans and livestock. Therefore, the development of innovative and effective therapies is essential for the treatment of *S. aureus*-induced severe infections. Ephedrine hydrochloride (EH) is a compound derived from ephedrine and is widely used for the management of cardiovascular diseases and hypotension. The results of our previous studies demonstrated that EH has anti-inflammatory activity in macrophages and protects against endotoxic shock. However, whether EH regulates the function of dendritic cells (DCs) and the immune response in *S. aureus*-induced infection is unknown. In this study, the anti-inflammatory and regulatory activity of EH on DCs was evaluated. EH increased the production of anti-inflammatory cytokine IL-10 and decreased the production of proinflammatory cytokines TNF-α and IL-12 in DCs stimulated with peptidoglycan (PGN), the main cell wall component in G^+ bacteria. The PI3K/Akt and p38 MAPK signaling pathways controlled EH-induced IL-10 expression and EH-inhibited TNF-α expression, respectively. The PGN-induced expression of co-stimulatory molecules CD40, CD80, CD86, and MHC class II molecule Iab was down-regulated in DCs by EH. Furthermore, EH protected the liver and kidney and increased the survival rate of mice with *S. aureus*-induced peritonitis. In conclusion, EH helps to keep immune homeostasis and alleviate organ damage during *S. aureus*-induced peritonitis. Therefore, EH may be a promising drug candidate in the treatment of *S. aureus*-induced severe infections and other invasive G^+ bacterial infections.

Keywords: Anti-inflammation, dendritic cells (DCs), ephedrine hydrochloride (EH), peptidoglycan, *staphylococcus aureus*

Introduction

Staphylococcus aureus (*S. aureus*) is a G^+ extracellular bacterium that causes infections in humans and livestock. No effective preventive vaccine against this pathogen is available to date. *S. aureus* can infect nearly all human tissues and causes a wide range of diseases, including skin and soft tissue infections, pneumonia, osteomyelitis, endocarditis, sepsis, and toxic shock syndrome [1-3]. *S. aureus* is the most common causative agent of severe invasive infections and sepsis in the hospital and community setting [3]. During severe bacterial infections, *S. aureus* activates the innate immune system and triggers the development of the systemic inflammatory response syndrome (SIRS), which is characterized by the increased expression of inflammatory cytokines. This bacterial species may also cause organ damage, sepsis, multiple organ failure, and even death [4, 5].

Different innate immune receptors, including Toll-like receptor (TLR) 2 and nucleotide-binding oligomerization domain-containing protein (NOD) 2, are involved in the recognition of *S. aureus* [6, 7]. TLR2 is expressed on the surface of many cells, including monocytes/macrophages and dendritic cells (DCs), which are acti-

vated by cell wall components of G^+ bacteria, e.g., peptidoglycan (PGN) and lipoteichoic acid (LTA). NOD2 recognizes PGN, which is transported into the cytosol and contributes to microbial surveillance [8]. TLR2 plays a vital role in host defense against *S. aureus* infection [9].

DCs and macrophages are recruited during *S. aureus* infection and play a critical role in recognizing pathogens, eliciting innate inflammatory response, and inducing the adaptive immune response. Upon activation by ligands from *S. aureus* or other G^+ bacteria (e.g., PGN), DCs secrete inflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), IL-12p70, and IL-10 [10]. After an initial hyper-inflammatory phase, DCs present bacterial antigens to T cells to evoke immune response and express co-stimulatory molecules, including CD40, CD80, and CD86 [11, 12]. IL-12p70 secreted by DCs is a crucial Th1/Th17 polarizing cytokine for inducing Th1 immune response [13]. The function of DCs in the treatment of *S. aureus* infection is controversial. DCs play a protective role against *S. aureus*, and the depletion of DCs in CD11c-DTR transgenic mice increases *S. aureus* load in the kidneys and lungs, resulting in severe inflammatory injury and mortality [14]. However, recent studies have shown that DCs also play a role in the worsening of atopic dermatitis by secreting high levels of IL-6, TNF-α, and IL-1β during secondary *S. aureus* infection [15]. To summarize, the balanced function of DCs is important for eliminating pathogens by eliciting a proper T cell response. However, the exacerbated response of DCs damages organs and worsens severe infection. Effective drugs for treatment of severe infections caused by *S. aureus* and other bacteria are necessary to modulate the function of DCs and reduce exacerbated immune responses. Inhibiting the excessive expression of inflammatory cytokines and decreasing DC-induced T cell overstimulation may be an effective method for treating sepsis, septic shock, and other conditions.

Ephedrine hydrochloride (EH) is a compound derived from ephedrine, which is obtained from *Ephedra sinica* (also known as Ma Huang, a traditional Chinese medicinal herb). Ephedrine acts as a α1- and β1-adrenergic agonist by increasing heart rate and blood pressure and is commonly used to treat hypotension induced by anesthesia, sympathectomy, or overdose of antihypertensive drugs [16, 17]. The results of our previous studies indicated that the anti-inflammatory and protective role of EH in lipopolysaccharide (LPS)-induced septic shock involved stimulating IL-10 production and inhibiting proinflammatory cytokine secretion [18, 19]. However, whether EH has a protective activity against *S. aureus*-induced infection *in vivo* is unknown to date.

In the present study, the anti-inflammatory role of EH in PGN-induced inflammatory response was demonstrated in DCs. Moreover, the protective activity of EH was determined in a *S. aureus*-induced peritonitis mouse model involved the reduction of the inflammatory response and damage to organs, and extension of the lifespan of infected mice. Therefore, EH is a promising drug candidate for treating severe infections caused by *S. aureus*.

Materials and methods

Mice and reagents

Female C57BL/6J mice from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China) were used at 4-5 weeks of age. Animal welfare and experimental procedures were carried out with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China) in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Phosphoantibodies against c-Jun N-terminal kinase/stress-associated protein kinase (JNK/SAPK, Thr183/Tyr185, Cat.4668, clone number 81-E11), the extracellular signal-regulated kinase p44/p42 (ERK1/2, Thr202/Tyr204, Cat.4370, clone number D13.14.4E), p38 (Thr180/Tyr182, Cat.4511, clone number D3F9), p65 subunit (Ser536) of NF-κB (Cat.3033, clone number 93H1), Akt (Thr308, Cat.2965, clone number C31E5E), Akt (Ser473, Cat.4060, clone number D9E), GSK-3β (Ser9, Cat.5558, clone number D85E12) were purchased from Cell Signaling Technology (Beverly, MA). The corresponding antibodies against JNK (Cat.9258, clone number 65G8), p44/42 MAPK (Erk1/2, Cat.4695, clone number 137F5), p38 MAPK (Cat.9212, batch number16), NF-κB p65 (Cat. 8242, clone number D14E12), Akt (Cat.4691, clone number C67E7), *β*-Actin Mouse mAb (Cat.3700, clone number 8H10D10), Anti-rabbit IgG, HRP-linked Antibody (Cat.7074) were purchased from Cell Signaling Technology (Be-

verly, MA). The inhibitors of different signaling pathways were obtained from Calbiochem (San Diego, CA). Dexamethasone (DXM) ($C_{22}H_{29}FO_5$), DMSO and thioglycolate were obtained from Sigma (St. Louis, MO). Ephedrine hydrochloride (EH) ($C_{10}H_{15}NO{\cdot}HCl$) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Peptidoglycan (PGN), the main component of the cell wall from *Staphylococcus aureus*, was purchased from Fluka (St. Louis, MO) and used at 25 μg/mL for all experiments *in vitro*. Recombinant mouse IL-4 and GM-CSF were obtained from PeproTech (London, United Kingdom). CD19 and CD4 monoclonal antibody-coated microbeads were purchased from Miltenyi Biotechnology (Bergisch Gladbach, Germany). Anti-mouse IL-10 neutralizing antibody (AB-417-NA) was purchased from R&D Systems (Minneapolis, MN).

Primary cell isolation and culture

Femurs and tibias of female mice (4 weeks old) were used to isolate bone marrow-derived dendritic cells (DCs), as described previously [20]. Splenic B or T cells were isolated from spleens by preparing a single-cell suspension depleted of red blood cells, and purified separately using CD19 or CD4 monoclonal antibody-coated microbeads as previously described [20].

Flow cytometry assay

DCs were treated with indicated concentrations of EH with or without PGN for 24 h, and then collected and labeled with Propidium iodide (PI) and Annexin-V-FITC provided by Calbiochem (San Diego, CA), following manufacturer's instructions [18, 19]. Samples were detected by BD Accuri™ C6 (BD, San Jose, USA) as described previously. DCs were labeled with fluorochrome-conjugated antibodies, including: anti-CD40 (clone number HB14), anti-CD80 (clone number 16-10A1), anti-CD86 (clone number B7-2) and anti-Iab (clone number 28-5-16S) antibodies (eBioscience) and detected by FACS. Flow cytometry data were analyzed and graphs were produced using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

Bacterial strains and acute peritonitis mouse model

Standard strain of *Staphylococcus aureus* (ATCC 6538) used in this study was obtained from ATCC (Manassas, VA) and used for *in vivo* assays in mice. *S. aureus* was grown in Luria-Bertani (1% Tryptone, 0.5% Yeast extract, 1% NaCl) medium which was agitated at 200 rpm in an incubator at 37°C. The optical density at 600 nm (OD600) of fresh suspension culture was measured using a BioTek Synergy 2 microplate readers and spectrophotometers (Vermont, USA). The density of culture was calculated according to the OD value. The bacterial suspensions were diluted with pre-warmed sterile PBS to give a final density of 1×10^9 CFU/mL. Inoculation was performed by intraperitoneal injection of 0.2 mL/mouse or 0.5 mL/mouse (LD80) to elicit the acute peritonitis mouse model [21]. Mice were pretreated with PBS or EH for 30 min and followed by intraperitoneal inoculation of *S. aureus*. The survival statuses of mice were recorded at different intervals up to 5 days. Blood samples were collected after 4 h and clotted for 3 h at 4°C. After centrifugation, serum samples were examined by ELISA for different cytokines. DXM (7 mg/kg body weight, equivalent to the clinical dose) was chosen as a positive control [18].

Hematoxylin and eosin (H&E) staining

S. aureus-induced peritonitis mouse model was carried out with C57BL/6J mice and different groups were treated as indicated. After 4 h, mice were sacrificed and livers and kidneys were collected to be fixed with 4% formaldehyde and embedded in paraffin. The slices of tissues were stained with by hematoxylin and eosin (H&E). Pictures of slices were taken by a Zeiss Imager M2 microscope as described [21].

Cytokine secretion measurement

Enzyme-linked immunosorbent assay (ELISA) kits for murine IL-10, TNF-α, IL-12p70 and IL-6 were purchased from R&D Systems (Minneapolis, MN). The cell supernatants were collected and tested by ELISA kit according to the manufacturer's instructions. The concentrations of cytokines in the serum samples from mice were also measured by ELISA.

Immunoblot analysis

DCs were stimulated by PGN and EH for different time courses and were lysed with M-PER™ Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Protein con-

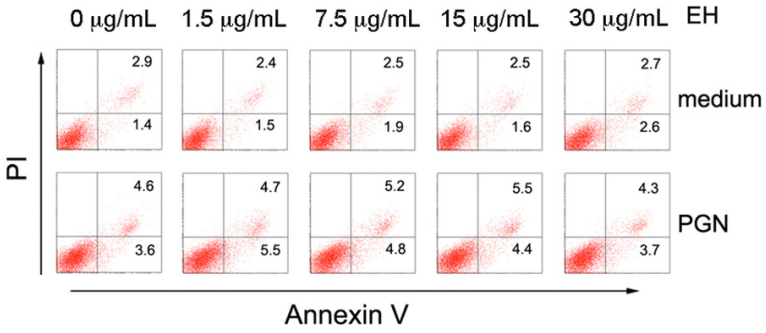

Figure 1. EH does not induce cellular apoptosis at the indicated concentrations in mouse DCs. Mouse BMDCs (2×10^5/300 μL) were plated in 24-well plates and stimulated by different concentrations of EH with or without PGN (25 μg/mL) for 24 h. Cells were then collected and labeled with Annexin V-FITC and PI, and analyzed by FACS. The clinical concentration of EH is 30 μg/mL. Similar results were obtained in three independent experiments.

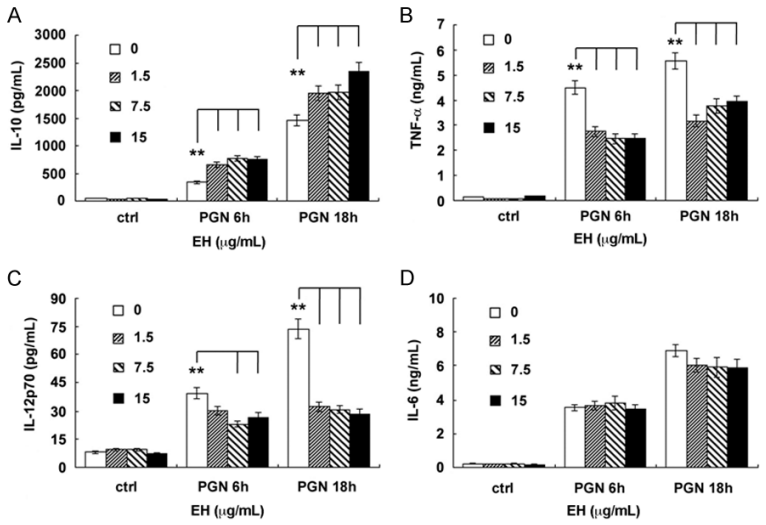

Figure 2. EH inhibits PGN-triggered inflammatory cytokine production and promotes IL-10 production in mouse DCs. DCs were cultured in conditional medium (RPMI 1640, containing 10% FBS, 10 ng/mL mGM-CSF and 1 ng/mL mIL-4). On day 5, they were plated (3×10^5/300 μL) in 24-well plates and were stimulated with PGN and different concentrations of EH. After 6 or 18 h, the cell supernatants were collected. The concentrations of IL-10 (A), TNF-α (B), IL-12p70 (C) and IL-6 (D) in the supernatants were measured by ELISA. Data are shown as mean ± SD of three independent experiments. **, P<0.01.

centrations were measured with bicinchoninic acid assay (Pierce, Rockford, IL) and were made equal with extraction reagent. Equal amounts of extracts were separated by 10% SDS-PAGE and transferred onto PVDF membranes for immunoblot analysis [21]. Actin was used as an internal control.

Statistical analysis

Results were given as mean ± standard deviation (SD) or mean ± standard error (SE). Statistical significance was determined with the two-tailed Student's *t*-test, with a *p* value of <0.05 or a *p* value of <0.01 considered statistically significant. Survival analysis were done using Log-Rank test. The survival curve was produced by Sigmaplot software.

Results

EH did not promote apoptosis in DCs

In our previous studies, we demonstrated that EH does not trigger apoptosis in macrophages and does not significantly affect cell viability of mouse peritoneal macrophages after LPS or PGN stimulation [18]. Considering that macrophages and DCs may have differential sensitivity to EH, apoptosis was examined in DCs by FACS using Annexin V and Propidium iodide (PI) labeling. EH (1.5-30.0 μg/mL) was added to the cell culture medium and cultured for 24 h with or without PGN stimulation (25 μg/mL). None of the measured EH concentrations induced detectable apoptosis in DCs (**Figure 1**).

EH promoted PGN-induced IL-10 secretion and inhibited the secretion of proinflammatory cytokines in DCs

PGN is the main component of the cell wall of Gram-positive bacteria and a ligand for TLR2, which induces inflammatory response in immune cells. DCs respond to PGN by secreting proinflammatory or anti-inflammatory cytokines. Therefore, the regulatory role of EH on the PGN-induced inflammatory response was examined in DCs. The ELISA results indicated that EH significantly decreased the PGN-induced secretion of TNF-α and IL-12p70 and stimulated PGN-induced IL-10 production in DCs. However, PGN-induced IL-6 secretion did not seem to be affected by EH treatment (**Figure 2**).

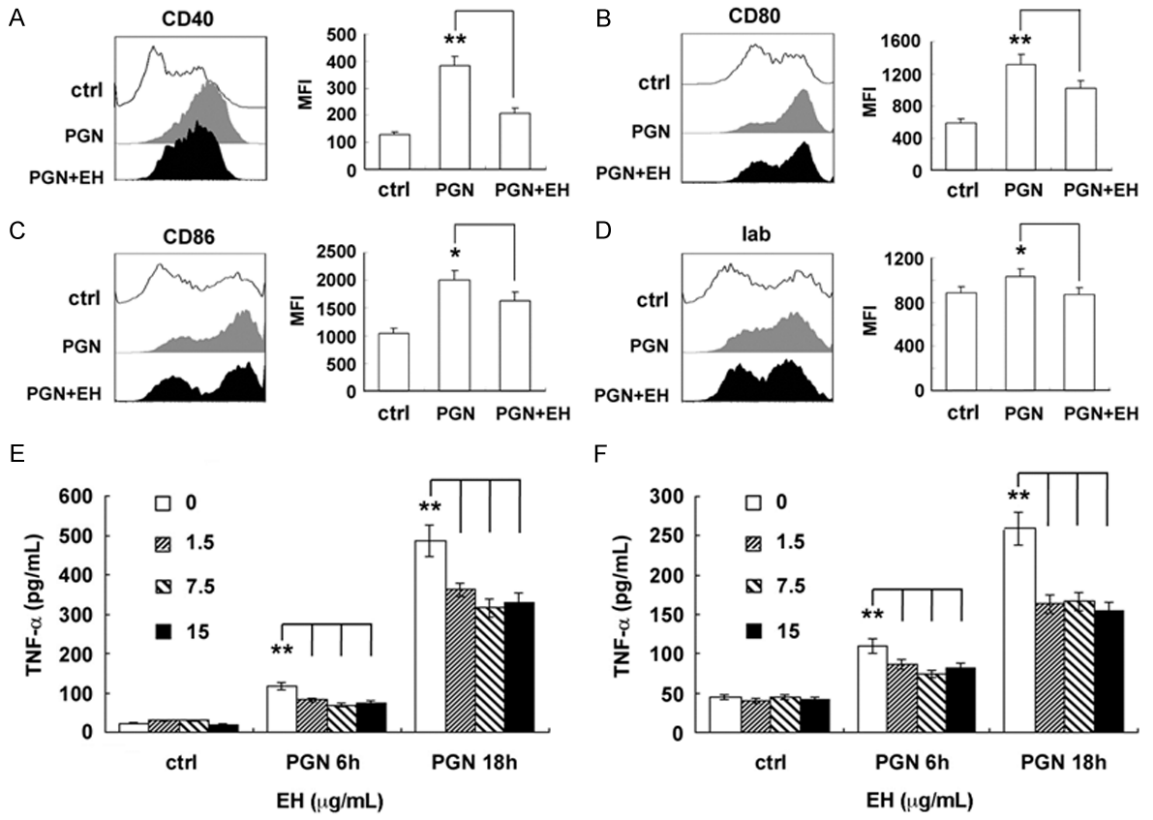

Figure 3. EH down-regulates co-stimulatory molecules in DCs and inhibits PGN-induced TNF-α production in T and B cells. (A-D) DCs were plated (3×10^5/300 μL) in 24-well plates and stimulated with or without PGN. After 18 h, DCs were harvested and analyzed for the expression of cell surface markers by FACS. The histograms at the right of each FACS figures were represented the values of mean fluorescence intensity (MFI). (E, F) Splenic T and B cells were enriched with anti-CD3 and anti-CD19 antibodies-coated microbeads respectively. T cells (E) or B cells (F) were stimulated with EH and PGN as indicated for 6 or 18 h, and the concentrations of TNF-α in the supernatants were measured by ELISA. Similar results were obtained in three independent experiments. Data are shown as mean ± SD. *, P<0.05; **, P<0.01.

EH reduced the expression of PGN-induced co-stimulatory molecules in DCs and inhibited the production of PGN-induced TNF-α in splenic T and B cells

DCs are known to be the most important antigen-presenting cells during bacterial infection. Antigen presentation to T cells by DCs during infection stimulates the expression of MHC class II molecules (e.g., Iab) and several co-stimulatory molecules, including CD40, CD80, and CD86. These molecules are important for subsequent T cell activation and adaptive immune response [11, 12]. As expected, PGN increased the expression of CD40, CD80, CD86, and Iab in DCs by mimicking bacterial infection (**Figure 3A-D**). Treatment with EH decreased the PGN-induced expression of CD40, CD80, CD86, and Iab on the cellular surface of DCs, and consequently reduced the T cell response in severe infections. The systemic response to bacterial infection is also related to the function of macrophages and T and B cells. As reported previously, EH inhibits the inflammatory response of macrophages upon PGN stimulation [19]. In addition to macrophages, T and B cells also contribute to the inflammatory response upon bacterial challenge. The effect of EH on the response of T or B cells was assessed by analyzing the secretion of TNF-α in the supernatant of splenic $CD4^+$ T and $CD19^+$ B cells using ELISA (**Figure 3E**, **3F**). The results indicated that PGN-induced TNF-α secretion was reduced by EH in T and B cells, demonstrating that EH directly inhibited the production of inflammatory cytokines by DCs and T/B cells, and decreased the expression of co-stimulatory molecules by DCs, leading to a subsequent decrease in the adaptive T cell response.

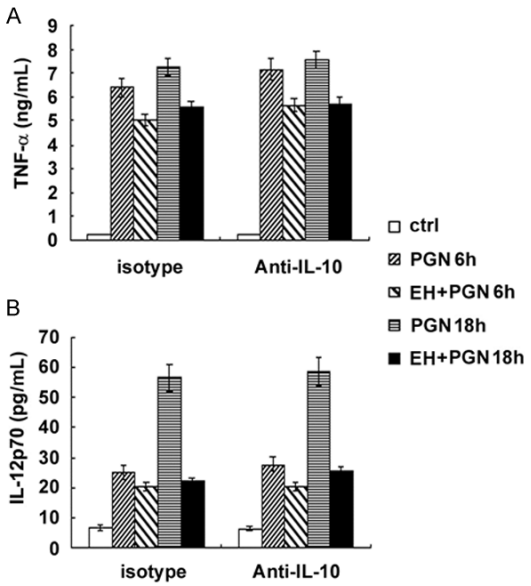

Figure 4. The down-regulation of TNF-α and IL-12p70 expression by EH is not dependent on the elevated IL-10 secretion. DCs (3×10^5/300 μL) were pre-treated with anti-IL-10 antibody (10 μg/mL) or isotype antibody for 30 min, and then were stimulated with PGN with or without EH (7.5 μg/mL) for 6 h or 18 h. The concentrations of TNF-α (A) and IL-12p70 (B) in the supernatants were examined by ELISA. Data are shown as mean ± SD of three independent experiments.

EH inhibited the production of proinflammatory cytokines in IL-10-independent manner

Anti-inflammatory cytokine IL-10 is known to inhibit the inflammatory immune response by decreasing the secretion of inflammatory cytokines and the expression of co-stimulatory molecules on DCs [22]. However, whether the decreased expression of TNF-α and IL-12p70 in DCs are caused by a secondary response to the EH-induced up-regulation of IL-10 is unknown. After treatment with isotype antibody or IL-10 neutralizing antibody (10 μg/mL), DCs were stimulated with PGN in the presence or absence of EH. The secretion of TNF-α and IL-12p70 in the supernatant was measured by ELISA after 6 h and 18 h of stimulation. The results indicated that pretreatment with IL-10 neutralizing antibody did not significantly affect the EH-inhibited secretion of TNF-α and IL-12p70 upon PGN stimulation (**Figure 4**), suggesting that EH regulates the expression of pro-inflammatory cytokines and IL-10 through different mechanisms.

EH inhibited PGN-induced p38 phosphorylation and promoted the activation of PI3K/Akt/GSK-3β pathway, leading to the regulation of TNF-α and IL-10

MAPKs (ERK, JNK, and p38 MAPK) and NF-κB signaling pathways regulate many cellular processes, including inflammation, survival, proliferation, differentiation, migration, and invasion. MAPKs also play an essential role in *S. aureus* infection and are activated by the ligation of PGN in TLR2 [23]. The molecular mechanisms underlying the anti-inflammatory activity of EH were further explored by analyzing the phosphorylation and activation of ERK, JNK, p38 pathways, and the p65 subunit of the NF-κB pathway by Western blot (**Figure 5A**, **5B**). The results indicated that EH treatment inhibited PGN-induced p38 phosphorylation but did not affect the PGN-induced phosphorylation of ERK, JNK, IKKα/β, and p65 of NF-κB. Phosphoinositide 3-kinase (PI3K)/Akt/GSK-3β signaling pathway also contributes to the production of IL-10 after TLR2 ligation [18, 24]. Our results demonstrated that EH treatment promoted the PGN-induced phosphorylation of Akt (Thr308) and phosphorylation of GSK-3β (Ser9) in DCs (**Figure 5C**). However, the phosphorylation of Akt (Ser473) did not seem to be affected by EH. In conclusion, EH inhibits p38 phosphorylation and enhances the activation of PI3K/Akt/GSK-3β pathway upon PGN stimulation.

The expression of IL-10 is regulated simultaneously by several signaling pathways in immune cells, including MAPKs, PI3K/Akt/GSK-3β, and NF-κB pathways, and TNF-α production is also regulated by multiple pathways [24]. The pivotal signaling pathways that contribute to the EH-mediated production of IL-10 and EH-inhibited production of TNF-α were also investigated. DCs were pretreated with DMSO, ERK inhibitor PD98059, JNK inhibitor SP600125, p38 inhibitor SB203580, PI3K inhibitor LY294002, or NF-κB inhibitor BAY11-7082 with or without EH for 30 min and then PGN was added to the culture medium for 6 h. The upregulation of the EH-mediated expression of IL-10 was abolished by pretreatment with LY294002 (**Figure 6A**). These results suggest that EH increases the PGN-induced expression of IL-10 by activating the PI3K/Akt/GSK-3β pathway. The inhibition of TNF-α expression by EH was

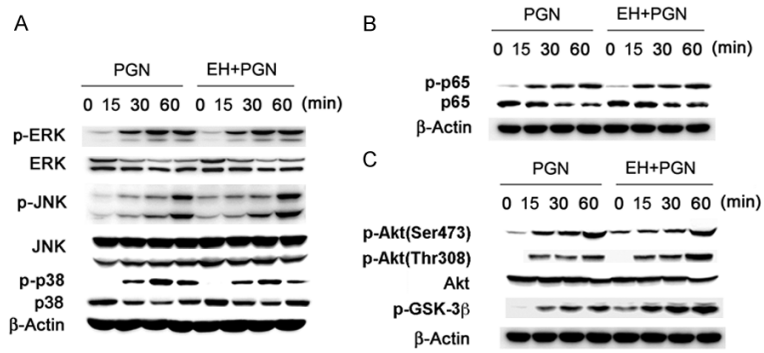

Figure 5. EH inhibits PGN-induced activation of p38 MAPK and promotes PI3K/Akt/GSK-3β signaling pathway in DCs. DCs (1×10^6/well) were plated in 6-well plates overnight and stimulated with PGN with or without EH (7.5 μg/mL) for 0, 15, 30 or 60 min. A. The expression of phospho-ERK (p-ERK), p-JNK, p-p38, and the corresponding total ERK, JNK and p38 MAPK were examined by Western blot. B. The expression of p-p65, total p65 and *β*-Actin were detected by Western blot. C. Phospho-Akt (p-Akt) on Ser473, Thr308 and the corresponding total Akt, p-GSK-3β (Ser9) and *β*-Actin were detected by Western blot. Similar results were obtained in three independent experiments.

abolished by pretreatment with SB203580 (a p38 inhibitor) but was not significantly affected in the groups treated with other inhibitors (**Figure 6B**), suggesting that EH decreases the PGN-induced expression of TNF-α by inhibiting the activation of p38 MAPK. In conclusion, EH promotes the PGN-induced expression of IL-10 by activating the PI3K pathway and inhibits TNF-α production through the inhibition of the p38 pathway.

EH ameliorated organ damage in mice infected with S. aureus

The anti-inflammatory role of EH in the PGN-induced inflammatory response was evaluated in DCs (**Figure 2**) and macrophage [18] *in vitro*. Furthermore, the anti-inflammatory role of EH in bacterial infections *in vivo* was evaluated in a *S. aureus*-induced peritonitis mouse model by the intraperitoneal injection of *S. aureus* in mice (2×10^8 CFU/mouse). Dexamethasone (DXM) is commonly used to treat sepsis or septic shock in the clinic and attenuate the expression of inflammatory cytokines, including IL-1β and TNF-α [19]. In the present study, DXM was chosen as a positive control to evaluate the anti-inflammatory effect of EH *in vivo*. EH at 27 mg/kg or 67.5 mg/kg was used in the peritonitis mouse model, and these two doses were equivalent to a dose of 3 μg/mL or 7.5 μg/mL in cellular assays. Consistent with the results observed *in vitro*, the secretion of IL-10 in sera was significantly increased by EH in a dose-dependent manner. In addition, EH had a synergistic activity with DXM in the upregulation of IL-10 expression (**Figure 7A**). A high dose (67.5 mg/kg) of EH alone or combined with DXM decreased serum TNF-α production in *S. aureus*-challenged mice (**Figure 7B**). In summary, EH increased serum IL-10 secretion and decreased serum TNF-α induced by *S. aureus in vivo*.

In patients with sepsis, the exacerbated expression of pro-inflammatory cytokines and chemokines may cause vasodilatation, vascular leakage, in addition to damage, dysfunction, or failure of organs such as the liver and kidney [4, 5]. The kidney and liver of the mice of each group were harvested after 4 h of *S. aureus* infection. The tissue sections were subjected to hematoxylin-eosin (H&E) staining. The histological changes observed in the kidneys of *S. aureus*-infected mice, including damage to the structure of kidney glomeruli and epithelial cells and the infiltration of inflammatory cells. Compared with the kidneys of *S. aureus*-infected mice, the histological changes observed in the animals from the EH group, DXM group, and the combined treatment group were less evident, and the glomerular structure was relatively normal. Similar to the histological changes in the kidneys, severe necrosis, impaired arrangement of hepatic plates, and inflammatory cell infiltration were also observed in the livers of *S. aureus*-infected mice. The histopathologic changes in the liver were ameliorated in the EH, DXM, and the combined treatment groups. These results indicate that EH can ameliorate the damage in the kidney and liver of mice with *S. aureus*-induced acute peritonitis, and this result is consistent with the anti-inflammatory changes in serum cytokine secretion.

EH protected mice from lethal S. aureus-induced peritonitis

The protective role of EH against *S. aureus*-induced lethal infection was assessed by evaluating animal survival rate. Different study

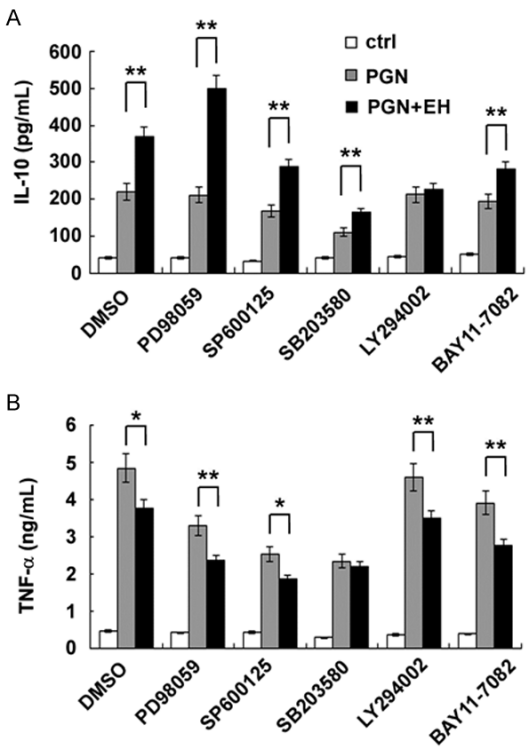

Figure 6. EH elevates IL-10 expression via enhancing PI3K/Akt/GSK-3β pathway and decreases TNF-α expression via inhibiting p38 phosphorylation in DCs. DCs (3×10^5/300 μL) were seeded in 24-well plates and were pretreated with DMSO, PD98059 (ERK inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), SB203580 (p38 inhibitor, 10 μM), LY294002 (PI3K inhibitor, 20 μM), or BAY11-7082 (NF-κB inhibitor, 10 μM) for 30 min. After that, PGN and EH (7.5 μg/mL) were added in the supernatants. After 6h, the concentrations of IL-10 (A) and TNF-α (B) in the supernatants were measured by ELISA. Data are shown as mean ± SD of three independent experiments. *, P<0.05; **, P<0.01.

groups, including those receiving PBS, a low or high dose of EH (27 mg/kg and 67.5 mg/kg), DXM, or EH + DXM were tested to determine the treatment that yielded the maximum protection in lethal *S. aureus*-induced peritonitis mouse model. The peritonitis mouse model was established by the intraperitoneal injection of a lethal dose of *S. aureus* (5×10^8 CFU/mouse). No mortality and no obvious adverse effects were observed in the PBS or EH-treated groups (without *S. aureus* infection) at the end of the observation period (5 days). Within 5 days, none of the peritonitis model mice (*S. aureus*-challenged mice) survived. However, low doses of EH treatment increased the survival rate to 75% at the end of the observation period compared with the peritonitis model group (P<0.01). Moreover, the survival of mice in the DXM-treated group was 37.5% (P<0.05), whereas the survival rate was increased to 75% in the group treated with a low dose of EH and DXM (P<0.01). Half of the mice in the group treated with a high dose of EH survived, and this survival rate was significantly higher than that of mice in the peritonitis model group (P<0.05) (**Figure 8**). Mice from the EH group and DXM + EH group showed the mild symptoms and improved quickly. These results indicated that EH protected mice against *S. aureus*-induced fatal peritonitis at all examined doses.

Discussion

S. aureus is the main bacterial species involved in wound infections [25]. The superficial infection caused by *S. aureus* may progress to invasive and even life-threatening infections, including bacteremia, pneumonia, and sepsis in some cases [26]. Several studies have reported that *S. aureus* infections are frequent in patients in intensive care units (ICUs), increasing hospitalization time and the burden on healthcare. Therefore, novel therapeutic strategies are necessary.

Vaccines against *S. aureus* are considered a suitable therapeutic strategy. However, despite the significant efforts in developing vaccines for *S. aureus*, the trial results have been disappointing [27-29]. To date, no licensed vaccines are available for *S. aureus* [30]. Research on vaccines for *S. aureus* is challenging for the following reasons: first, *S. aureus* is an extracellular pathogen but can survive in different types of non-professional phagocytes, such as endothelial and epithelial cells [31, 32]. Second, *S. aureus* has a high genetic variability; for this reason, a multivalent vaccine may be more effective than a monovalent vaccine [33]. Third, healthy carriers have a robust antibody response to *S. aureus*, and the antibodies elicited by the vaccine do not reliably protect against different *S. aureus* strains [34]. Although the 4-antigen *S. aureus* vaccine SA4Ag was well tolerated and induced high levels of bacteria-killing antibodies in healthy adults in a phase I study [30], the efficacy of this antibody-based vaccine in later clinical trials is questionable because many patients develop natural antibodies against *S. aureus* but the pathogens cannot be excluded or decolonized.

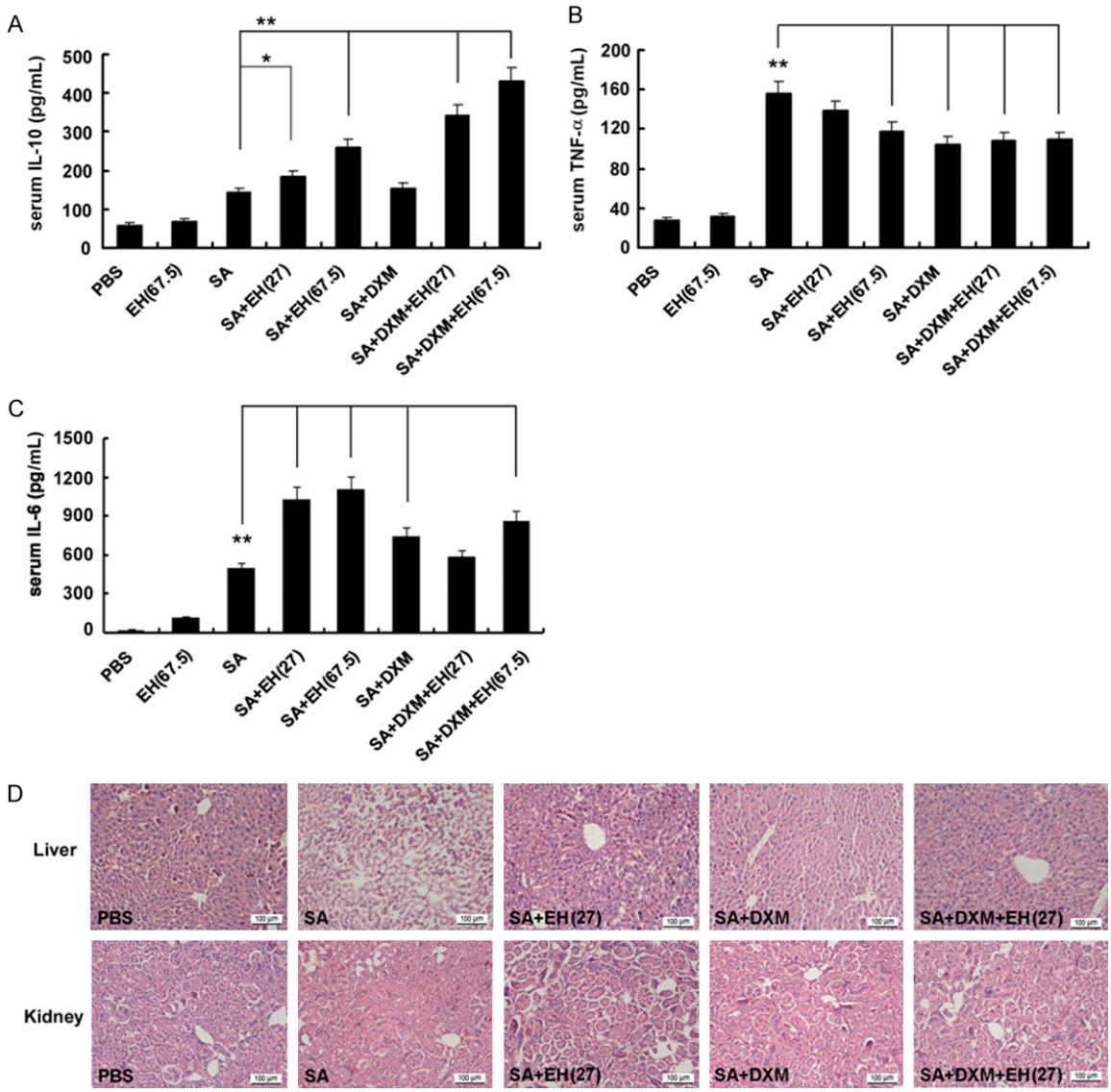

Figure 7. EH decreases the inflammatory response and alleviates organ damage of mice after *Staphylococcus aureus* challenge. C57BL/6J mice were randomly divided into 8 groups (n = 5): PBS group (0.2 mL/mouse), EH group (67.5 mg/kg), *S. aureus* (SA, ATCC 6538) group, SA + EH (27 mg/kg) group, SA + EH (67.5 mg/kg) group, SA + DXM (7 mg/kg) group, SA + DXM (7 mg/kg) + EH (27 mg/kg) group and SA + DXM (7 mg/kg) + EH (67.5 mg/kg) group. All groups of mice were pre-treated with PBS, EH and/or DXM for 30 min respectively, and then were challenged by intra-peritoneal infection of *S. aureus* (2×10^8 CFU/mouse). Mice were sacrificed after 4 h and blood samples were clotted for 3 h at 4°C. Serum samples were prepared by centrifugation at 200 g ×20 min. The concentrations of IL-10 (A), TNF-α (B) and IL-6 (C) were detected by ELISA. Data were shown as mean ± SE (standard error) of 5 mice per group. *, $P<0.05$; **, $P<0.01$. (D) The liver and kidney from each group were harvested after 4 h of inoculation of *S. aureus* for H&E staining (×200). N = 3-5/group. SA represents *S. aureus*.

In addition to vaccination, two other therapeutic strategies are available for treating diseases caused by *S. aureus* [35]. The first strategy includes the use of antibiotics to kill the pathogen directly. However, antibiotic resistance is common in some *S. aureus* strains (e.g., methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus*), and the available antibiotics present little efficacy against these strains [36, 37]. The second strategy involves the use of antibodies or natural products to modulate the host's immune response. In *S. aureus*-induced severe infections, an exacerbated inflammatory response may cause SIRS and subsequent sepsis. TNF-α, IL-6, and IL-12 secreted by DCs and macrophages are known to be actively

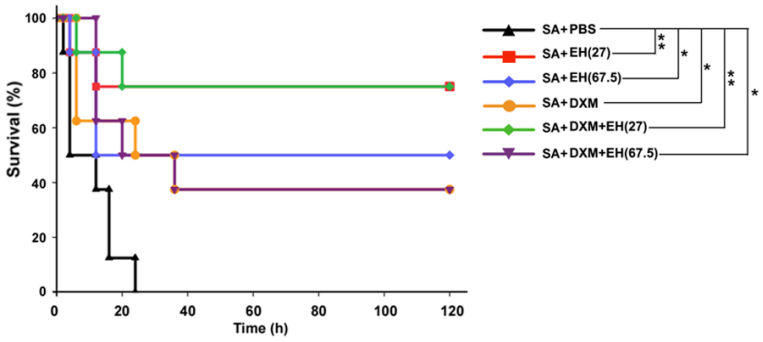

Figure 8. EH protects mice from lethal *S. aureus* infection. C57BL/6J mice were divided into 8 groups (n = 8): PBS group (0.2 mL/mouse), EH group (67.5 mg/kg), *S. aureus* (SA, ATCC 6538) group, SA + EH (27 mg/kg) group, SA + EH (67.5 mg/kg) group, SA + DXM (7 mg/kg) group, SA + DXM (7 mg/kg) + EH (27 mg/kg) group and SA + DXM (7 mg/kg) + EH (67.5 mg/kg) group. All groups of mice were pre-treated with PBS, EH and/or DXM for 30 min respectively, and then were challenged by a lethal dose of *S. aureus* (5×10^8 CFU/mouse) intraperitoneally in corresponding groups. The survival data was recorded in the following 5 days. Data were analyzed using Log-Rank test and survival curve was generated by Sigmaplot software. SA represents *S. aureus*. *, P<0.05. **, P<0.01.

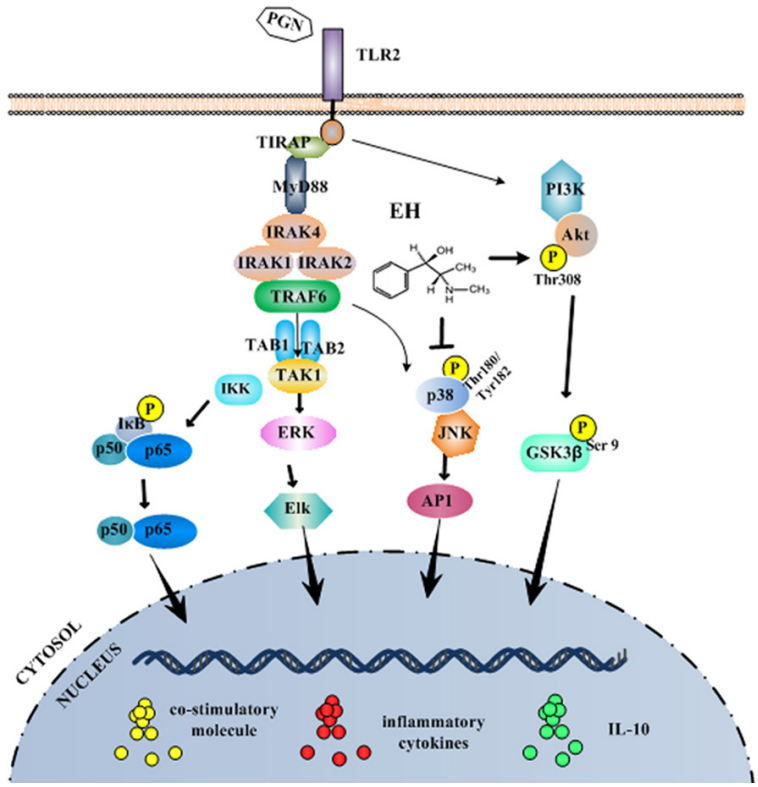

Figure 9. EH regulates PGN-induced inflammatory response in DCs. EH inhibits PGN-induced activation of p38 MAPK and promotes PI3K/Akt/GSK-3β signaling pathway in DCs, regulating the downstream cytokine expression.

involved in the inflammatory immune response of the host. TNF-α can induce the infiltration and activation of neutrophils and upregulate the expression of adhesion molecules in immune cells. TNF-α is also responsible for the initial hypothermia and lethality of sepsis in mice [38]. IL-6 is a key mediator of fever in mice during sepsis [38]. IL-12 is a pivotal regulatory cytokine that preferentially activates NK and Th1 cells to produce IFN-γ and other immune factors. Although the use of proinflammatory cytokine neutralizing antibodies are effective against sepsis in the clinic [39, 40], the use of one or two neutralizing antibodies to antagonize all cytokine types during bacterial infection is expensive and partially effective. Therefore, we believe that developing natural products to modulate the immune response of the host against *S. aureus* or even antibiotic-resistant *S. aureus* infection rather than single neutralizing antibody or a combination of antibodies is a promising strategy. Active ingredients from natural products are expected to control the exacerbated and harmful inflammatory response and keep a balance between the inflammatory and anti-inflammatory response to *S. aureus* [21]. Furthermore, natural products are much cheaper than the use of mixed antibodies against inflammatory cytokines.

In the past few years, many studies have revealed that traditional Chinese medicine is a tremendous source of new drug candidates to regulate *S. aureus*-induced inflammatory responses. Puerarin has anti-inflammatory activity against *S. aureus* infection by inhibiting NF-κB and MAPK activation [36]. Brazilin has anti-inflammatory activity for *S. aureus*-induced mastitis in mice by regulating TLR2 and its associated signaling path-

ways [41]. Piperine, an active plant alkaloid obtained from black pepper, is another option for the treatment of *S. aureus* infection [42]. In our previous studies, EH had anti-inflammatory function in endotoxic shock. EH protects mice against LPS challenge by decreasing the expression of LPS-induced inflammatory cytokines and increasing the expression of IL-10 [19]. We also demonstrated that EH stimulated the production of IL-10 and inhibited the production of IL-6 via the PI3K/Akt pathway after PGN stimulation in macrophages [18]. The results of the present study indicated that EH inhibited the production of proinflammatory cytokines (IL-12 and TNF-α) and promoted IL-10 expression in PGN-stimulated DCs, and might decrease exacerbated Th1 responses by down-regulating the expression of co-stimulatory molecules CD40, CD80, CD86, and the MHC class II molecule Iab. The macrophages and dendritic cells have their respective roles in generating an innate and adaptive immunological response in *S. aureus*-induced peritonitis. The peritoneal dendritic cells are far less comparing to the macrophage in number; however, DCs also have an important role in the immune system during the bacteria-induced peritonitis. Similar to macrophages, DCs can up-regulate genes leading to expression of various cytokines and chemokines involved in the inflammatory and immune response. What's more, DCs play a critical role in the initiation of cell mediated, Th1-type immune responses by uptaking antigens from the pathogen and subsequently migrating to nearby secondary lymphoid tissues to activate T cells. Regulating the function of DCs could be a good therapeutic strategy against *S. aureus* infection.

During *S. aureus* infection, Toll-like receptor 2 (TLR2) is the main receptor involved in the identification of pathogen-associated molecular patterns, including PGN and LTA, and activation of ERK, JNK, p38, PI3K/Akt/GSK-3β, and NF-κB pathways, lead to the expression of proinflammatory cytokines and IL-10 [23]. The overexpression and activation of Akt promote IL-10 expression upon LPS or PGN stimulation [21, 43]. Here we demonstrated that EH promoted PGN-induced phosphorylation of Akt (Thr308) and downstream GSK-3β (Ser9), and the pretreatment with PI3K inhibitor (LY294002) abolished the increase in the expression of IL-10 by EH. However, EH inhibited PGN-induced p38 phosphorylation and the pretreatment with a p38 inhibitor abolished EH-inhibited TNF-α secretion. Therefore, EH increased the PGN-induced expression of IL-10 and decreased the expression of inflammatory cytokines (e.g., TNF-α) in DCs through the PI3K/Akt/GSK-3β and p38 pathways, respectively (**Figure 9**).

Nevertheless, increasing evidence demonstrates that *in vivo* immune responses to *S. aureus* can differ substantially from observations *in vitro* [44-46]. A peritonitis mouse model was developed by injecting *S. aureus* intraperitoneally in mice to assess the anti-inflammatory and protective role of EH *in vivo*. EH increased IL-10 production and decreased TNF-α production in sera, and improved liver and kidney damage, consequently protecting *S. aureus*-infected mice. IL-6 expression was not affected by EH treatment in vitro. And *S. aureus*-induced IL-6 secretion was increased in mice treated with EH or DXM. The early anti-inflammatory and immunosuppressive responses triggered by DXM or EH treatment in this mouse model may decrease chemokine expression and inhibit the early chemotaxis of neutrophils or monocytes/macrophages to engulf and digest bacteria, which in turn may result in a temporary increase in bacterial burden, and this temporary increase may lead to an increase in IL-6 secretion. However, this hypothesis needs to be confirmed by future studies.

EH is an activator of the α1-adrenoreceptor and β1-adrenoreceptor and is widely used in the treatment of hypotension by increasing blood pressure and heart rate. In this experiment, the blood pressure-boosting effect of EH provided additional protection to mice suffering from hypotension caused by *S. aureus*-induced septic shock. In the present study, the preventative experiment was designed to evaluate the potential protective effects of EH. The therapeutic experiment using combination of antibiotics and EH may be promising and practical. Antibiotics and EH are expected to play a role in pathogens and the host simultaneously, and to accelerate the recovery of the host from infection by decreasing the excessive secretion of inflammatory cytokines and reducing the bacterial burden.

Our results demonstrated anti-inflammatory role of EH on the PGN-induced immune response in DCs by regulating of PI3K and p38 pathways. EH also play the protect mice from organ dam-

age and mortality caused by *S. aureus*. EH might be an effective drug candidate in the treatment of *S. aureus*-induced severe infection. Further studies are needed to investigate the combination of EH and antibiotics against *S. aureus*.

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Disclosure of conflict of interest

None.

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