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Quercetin reduces *Streptococcus suis* virulence by inhibiting suilysin activity and inflammation

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ABSTRACT

Streptococcus suis, a globally distributed bacterial pathogen, is an important zoonotic agent for humans and animals that can lead to multiple deaths and cause major economic losses. Suilysin (SLY), secreted by most pathogenic *S. suis* strains, is a cytotoxic toxin that belongs to the cholesterol-dependent cytolysin family; this toxin plays a key role in a mouse meningitis model, suggesting that effective interference with the biological activity of SLY may be a potential treatment for *S. suis* infection. In addition, the inflammatory response induced by *S. suis* is an important manifestation in infections and is associated with multiple fatal diseases. In this study, we found that the natural compound quercetin can directly inhibit the pore-forming activity of SLY without affecting bacterial growth and SLY secretion at the concentrations tested in our assay. In addition, quercetin treatment significantly alleviated cytotoxicity caused by *S. suis* infection and effectively reduced the release of the pro-inflammatory cytokines IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) stimulated by bacteria. Significantly decreased mortality was observed for the *S. suis*-infected mice that received quercetin. Our results suggested that quercetin may represent a promising therapeutic candidate for *S. suis* infection by targeting SLY and the subsequent inflammation. The present study provides a new strategy and leading compound for *S. suis* infection.

1. Introduction

Streptococcus suis (*S. suis*), one of the most important pathogens in the swine industry, is a gram-positive bacterium commonly found worldwide that can cause major economic losses and several fatal infections, including streptococcal toxic shock syndrome (STSS), meningitis and arthritis [1–3]. In the United States alone, this bacterium has resulted in the loss of hundreds of millions of dollars per year [4]. *S. suis* is considered an emerging and serious threat to public safety, as it is an opportunistic zoonotic pathogen [3]. To date, more than 30 serotypes of *S. suis* have been identified based on the specificity of the capsule antigens [5], and serotype 2 is the most frequently isolated serotype from clinically infected pigs worldwide [6,7];most meningitis cases are caused by *S. suis* type 2 (SS2) [5]. This bacterium poses a major hazard to pig producers and pork processors who have close contact with pigs and pork products or consumers who eat undercooked pork [5,8]. Approximately 1650 cases of human *S. suis* infections were reported worldwide before 2014 [9]. In the southeast Asian countries Vietnam and Thailand, *S. suis* infection is one of the leading causes of bacterial meningitis in adults [10,11]. In 1998 and 2005, two severe outbreaks of *S. suis* occurred in China. Hundreds of people were infected, and dozens of deaths occurred in a short period of time, which caused a major public health crisis, incalculable economic losses and serious consequences [12,13]. *S. suis* infections have caused serious public health issues, and effective treatment methods and strategies are urgently needed.

More than half of Chinese patients infected by SS2 in the aforementioned outbreak in 2005 had an unusual clinical manifestation of STSS, including disseminated intravascular coagulation, which was associated with platelet-bacterium interactions, and these patients showed increased mortality [13–15]. Suilysin (SLY), a secreted toxin of most virulent *S. suis* strains, was reported as the sole stimulatory factor for platelet activation and aggregation in the infections caused by *S. suis*. Moreover, the SLY-negative SS2 strain 1330 could not induce platelet aggregation [16], suggesting SLY may play a crucial role in DIC of STSS induced by SS2. SLY is an important exocrine toxin of SS2, a member of the cholesterol-dependent cytolysin (CDC) family, which can create pores in cholesterol-containing membranes and then lead to cell lysis [17,18]. The role and mechanism of SLY in human infections with SS2 are still unclear; however, the worldwide clinical case records

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show that SLY-positive strains led to more severe symptoms in China and southeast Asia than SLY-negative strains in North America [10,19,20]. In addition, Takeuchi et al. found that *S. suis* ST1, a strain producing high levels of SLY, caused meningitis more often than strains that produced low levels of SLY or no SLY [21]. In addition, the SS2 strain 05ZYH33 with increased SLY production showed hypervirulence in vivo and in vitro compared to *S. suis* 1940, a strain with lower levels of SLY production, which had a higher ability to penetrate the epithelium and survive in the bloodstream [22]. Moreover, SLY could also stimulate the release of heparin binding protein, induce inflammatory responses and increase the vascular permeability in mice [23]. Furthermore, SLY played an important role in a mouse model of SS2 meningitis. The mice infected with high levels of the SLY-producing strain had survival rates of less than 10%; however, in another group of mice used as a control, which was infected with a low level of the SLY strain, or a sly knockout strain, only a few deaths were observed, and mortality decreased significantly. Compared with the group infected with the strain producing high levels of SLY, the control group showed significantly reduced colony colonization and inflammation in the brain. These results further demonstrated that SLY was a crucial factor contributing toSS2 meningitis [21]. Thus, SS2 requires a series of virulence factors to successfully infect and cause host disease; SLY plays a crucial role in this process and may be related to STSS and meningitis. Therefore, SLY could be a potential new target for the treatment of SS2 infection.

S. suis infection can trigger a series of severe inflammatory reactions, especially arthritis and meningitis [5]. An excessive inflammatory response is a manifestation of STSS induced by SS2 [24]. Several studies have suggested that the increasing permeability through the blood-brain barrier during SS2 infection was stimulated by inflammatory reactions or mediators that occurred during adhesion and infection [25]. In addition, SS2 could stimulate monocytes/macrophages and produce pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α and monocyte chemotactic protein-1 (MCP-1) [26,27]. In addition, SS2 infection results in recognition of toll-like receptor 2 (TLR 2) and activation of members of the mitogen-activated protein kinase (MAPK) pathway, including c-Jun N-terminal kinase (JNK) and p38MAPK extracellular-signal-regulated kinase (ERK), which are closely related to the inflammatory response and can also regulate cell differentiation, proliferation and death [28,29]. A series of inflammatory reactions induced by SS2 are also an important part of the pathogenic process. Therefore, attenuating the inflammatory response of SS2 may be an important strategy for the treatment of SS2 infection.

Antimicrobial therapy is currently the main method for clinical treatment of SS2 infection [30], but bacterial resistance is becoming increasingly serious, especially given that the rate of development of new antibiotics is much lower than the emergence of bacterial resistance [31]. To effectively improve or eliminate the current unfavorable situation in the fight against *S. suis* infection, new anti-infective strategies and agents are urgently needed. In recent years, anti-virulence strategies have been recognized as promising and effective approaches to fight the rapid development of drug resistance [32]. Targeting virulence factors does not affect the normal growth of bacteria [33] and does not put a strong survival selection pressure on bacteria, which may delay the emergence of drug resistance. However, treatment with anti-virulence agents could significantly reduce the pathogenicity of pathogens [32,33]. At present, anti-virulence strategies using specific antibodies against α-hemolysin can significantly inhibit the virulence of *S. aureus* in vitro and in vivo [34].

Quercetin is a natural flavonoid that can be extracted from a variety of plants and fruits and has various pharmacological and biological effects, including antiviral, antioxidation, anticancer and anti-inflammatory effects [35–38]. Recent studies have found that quercetin could inhibit obesity and obesity-induced inflammation in cells and mice, which could also inhibit angiogenesis in nasopharyngeal carcinoma [39,40]. In addition, quercetin induced the differentiation of bone marrow mesenchymal stem cells [41]. However, the effect of quercetin in infections caused by SS2 and the interaction with SLY have not been reported thus far. In this study, we demonstrate that the natural bioflavonoid compound quercetin could inhibit the activity of SLY by preventing SLY oligomerization and significantly reduce the inflammatory response in SS2 infected cells and the mortality in infected mice. Thus, our results suggested quercetin may be a promising therapeutic candidate to treat SS2 infections.

2. Materials and methods

2.1. Bacterial strain and regents

The strain used in this study was the highly virulent SS2 ZY05719, which was isolated from pigs and kindly provided by Professor Hongjie Fan from Nanjing Agricultural University (Nanjing, China). Cell culture medium (Dulbecco's modified Eagle's medium, DMEM) was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Biological Industries (BI). The bacterial medium Todd-Hewitt broth (THB) was obtained from Qingdao Hope Bio-Technology (Qingdao, China). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich. Quercetin was purchased from Chengdu Herbpurify (Chengdu, China). The Cytotoxicity Detection Kit (LDH) was purchased from Roche Basel (Switzerland). The live/dead reagent was from Invitrogen. IL-1β, IL-6, and TNF-α ELISA kits were obtained from Biolegend (CA, USA).

2.2. Cell infection

SS2 was cultured in THB with 2%yeast extract and 5% FBS at 37 °C to an OD = 0.8 (mid-logarithmic growth), centrifuged at 3000 rpm for 5 min, washed twice by PBS and resuspended in complete DMEM without FBS or PBS.

J774 cells were cultured in complete DMEM containing 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 5% CO_2 and seeded in 96-well plates at a concentration of 2×10^4 cells per well for cytotoxicity assays and in 6-well plates at a concentration of 1×10^6 cells per well for ELISAs and western blotting analysis. The cells were infected with SS2 for 5 h at 37 °C.

2.3. Determination of minimal inhibitory concentration and growth curve

SS2 in mid-logarithmic growth stage was adjusted to a bacterial concentration of 5×10^5 colony-forming units (CFUs)/mL. The next procedures followed the JL Watts guidelines M31-A3 [42] to measure the minimal inhibitory concentration (MIC) of quercetin for SS2. For measurement of the growth kinetics of SS2 with quercetin, the bacterial culture in logarithmic growth phase was further cultured with different doses of quercetin to a final concentration of 0, 4, 8, 16, and 32 μg/mL, and bacterial densities at OD_{600nm} were monitored every 30 min for 6 h.

2.4. Hemolysis assay

Overnight culture of SS2 was transferred (1:50) to THB with 2% yeast extract and 5% FBS and treated with different doses of quercetin (0, 4, 8, 16, and 32 μg/mL) and then further cultured to until the optical density at 600 nm (OD_{600}) reached 2.4–2.6. The above cultures were centrifuged to collect the supernatants, and 100 μL of the supernatant was added each sample with 875 μL phosphate buffered saline (PBS) and 25 μL defibrinated rabbit blood, followed by a gentle mix and incubation for 35 min at 37 °C. A sample with PBS or ddH_2O was used as a negative or positive control, respectively. After the incubation, each sample was centrifuged to obtain the supernatant, and the optical density at OD_{543} was measured.

In addition, purified SLY protein was obtained as previously described [43]; 100 μL purified proteins (100 ng/mL) were suspended in a system with 875 μL PBS, 25 μL defibrinated rabbit blood and different

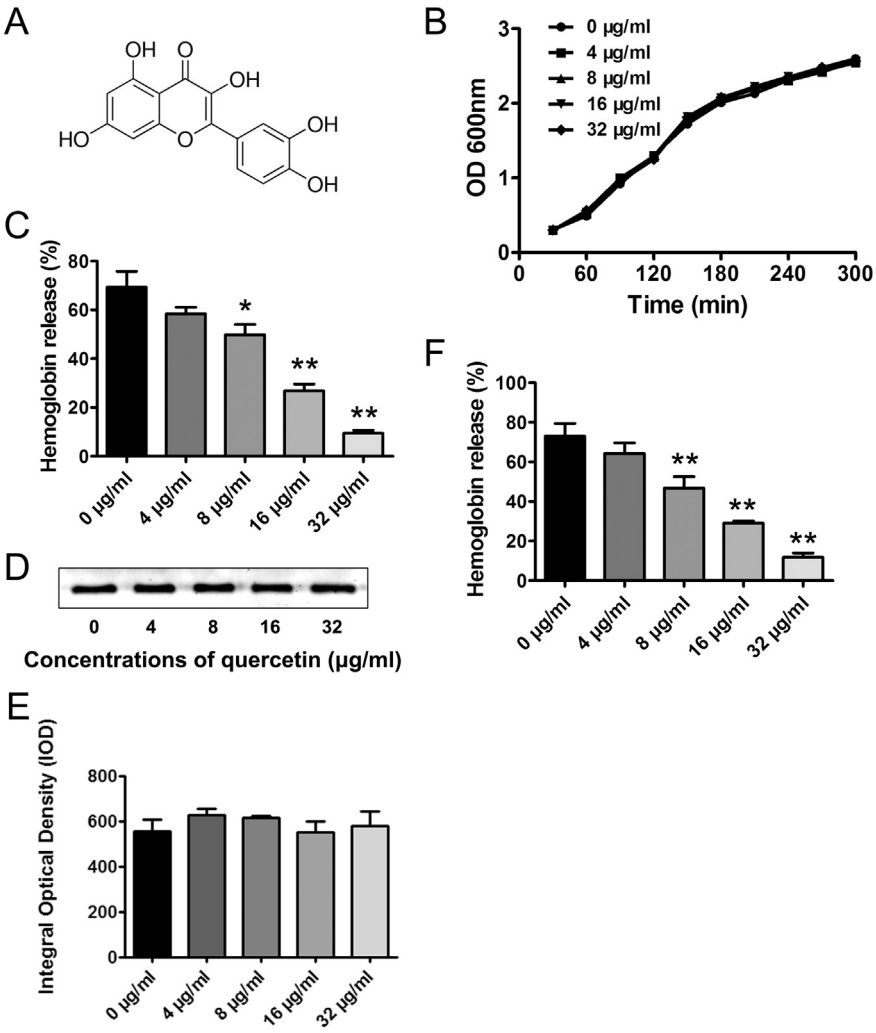

Fig. 1. Inhibition of suilysin-induced pore-forming activity by quercetin. A Chemical structure of quercetin. B SS2 was incubated Todd-Hewitt broth with 2% yeast extract and 5% FBS at different concentrations of quercetin, and the growth of each sample was monitored every 30 min at OD_{600}. C Inhibition of the pore-forming activity in the culture supernatant of SS2 by quercetin. SS2 was co-cultured with quercetin, and the hemolytic activity of the supernatants was analyzed by a hemolysis assay. The value at OD_{543} of each supernatant in the reaction sample incubated with rabbit blood was compared to the control group treated with ddH_2O. D and E The expression of SLY in bacterial culture supernatant with various concentrations of quercetin was detected by western blot. F Dose-dependent inhibition of the activity of purified SLY by quercetin. Purified SLY was treated with the indicated concentrations of quercetin, and the hemolytic activity was determined as described in panel C. Data are shown as the mean ± SD from more than 3 independent experiments. $^{*}P < .05$ and $^{**}P < .01$, by 2-tailed Student's *t*-tests.

final concentrations of quercetin. Other operations were performed as described previously.

2.5. Oligomeric inhibition assay

The purified SLY protein was adjusted to 0.4 mg/mL with PBS and incubated with various concentrations of quercetin for 30 min at 37 °C, followed by another incubation with saturated potassium chloride at 37 °C for 5 min. Then, 2.5 μL rabbit red blood cells were added to each sample for an ice bath for 5 min. After addition of SDS-PAGE loading buffer without 2-hydroxy-1-ethanethiol in a bath for 10 min at 55 °C, the protein sample was separated by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes The protein of interest (SLY monomer and oligomer) was visualized with primary anti-SLY rabbit serum (Tianjin Sungene Biotech Co., Ltd., Tianjin, China) [43] diluted 1:1000 and secondary antibody (horseradish peroxidase-conjugated anti-rabbit, Sigma-Aldrich) diluted 1:8000 using the Amersham ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

2.6. Western blotting analysis

A total of 20 μL bacterial culture supernatants as described above were mixed with SDS-PAGE loading buffer, boiled for 8 min and then separated on a 12% SDS-PAGE gel. The SLY was detected as described in the oligomeric inhibition assay.

Total proteins from J774 cells infected with SS2 (multiplicity of infection [MOI] = 8:1 for 5 h) were extracted by M-PER Mammalian Protein Extraction Reagent (Thermo) and separated by 10% SDS-PAGE. The primary antibodies for p38, p-p38, ERK1/2, p-ERK1/2, NF-κB p65 and β-actin (Proteintech) diluted 1:1000 and the secondary rabbit or mouse antibody diluted 1:10000 were used for the detection of signaling proteins as described for the oligomeric inhibition assay. The intensity was measured using Image-Pro Plus software.

2.7. ELISAs

The levels of inflammatory cytokines in cell-free supernatants from the J774 cell and SS2 co-culture system (MOI = 8:1 for 5 h) were measured by IL-1β, IL-6, and TNF-α ELISA kits according to the instructions.

2.8. Cytotoxicity assay

The J774 cells were infected with SS2 treated with different concentrations of quercetin at an MOI of 12. The cells treated with DMEM or 2.5 vol% Triton X-100 were set as the negative or positive control. Following infection for 5 h, the Cytotoxicity Detection Kit was used to measure the release of LDH in the supernatants of the co-culture system. Furthermore, a confocal laser scanning microscope (Nikon, Tokyo, Japan) was used to obtained microscopic images of cells stained with live/dead (green/red) reagent (Invitrogen).

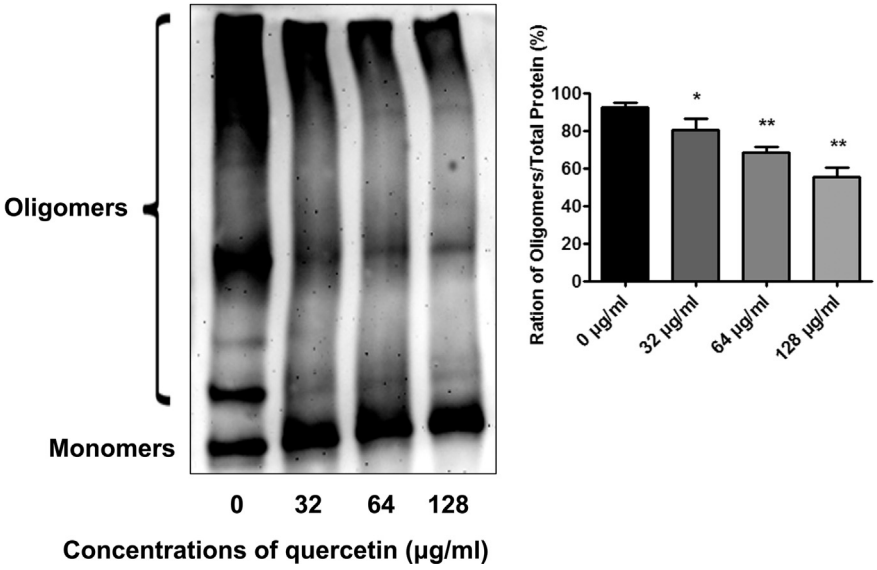

Fig. 2. Inhibitory effect of quercetin on oligomerization of suilysin. Purified SLY was incubated with various doses of quercetin, and the oligomerization of SLY was detected by western blot analysis.

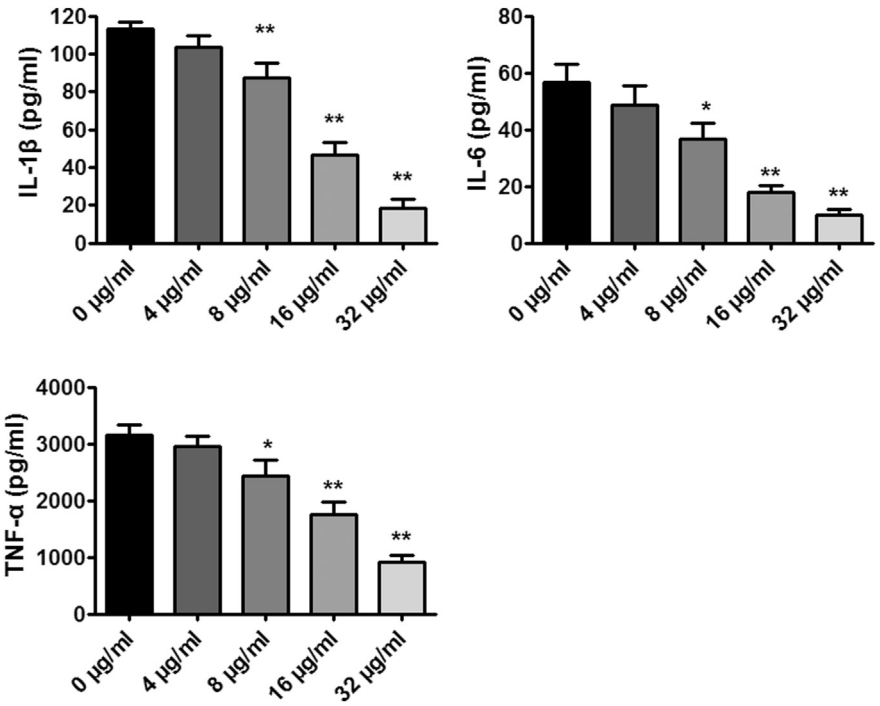

Fig. 3. Inhibitory effects of quercetin on SS2-mediated production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in host cells. J774 cells were infected with SS2 with various concentrations of quercetin (MOI = 8:1) for 5 h, and the release of IL-1β, IL-6 and TNF-α in cell-free supernatant was measured by ELISAs. Data are shown as the mean ± SD from more than 3 independent experiments. $^{*}P < .05$ and $^{**}P < .01$, by 2-tailed Student's *t*-tests.

2.9. The mouse model infected with SS2

All the animal experiments were approved by and conducted in accordance with the guidelines of the Animal Care and Use Committee of Jilin University. The 6-week-old female C57BL/6J mice (Experimental Animal Center of Jilin University, Changchun, China) were intraperitoneally inoculated with 1×10^8 CFUs per mouse. The quercetin was dissolved in DMSO to the final concentration of 80 mg/mL and the infected mice subcutaneously received 25 μL of quercetin (100 mg/kg) at 2 h post-infection and every 8 h or 25 μL of DMSO at the same schedule as the control. The survival of each group ($n = 30$) was monitored for 96 h.

2.10. Statistical analysis

SPSS 13.0 statistical software was used to perform all the statistical analysis, and independent Student's *t*-tests were analyzed for significance, with the significance level set at 0.05.

3. Results

3.1. Quercetin inhibits SLY pore-forming activity without interfering with SS2 viability

The MIC of the natural flavonoid quercetin (Fig. 1A) on SS2 was more than 1024 μg/mL; additionally, no significant difference was observed for the growth of SS2 in the presence of various concentrations of quercetin (Fig. 1B). These data suggested that quercetin had no anti-

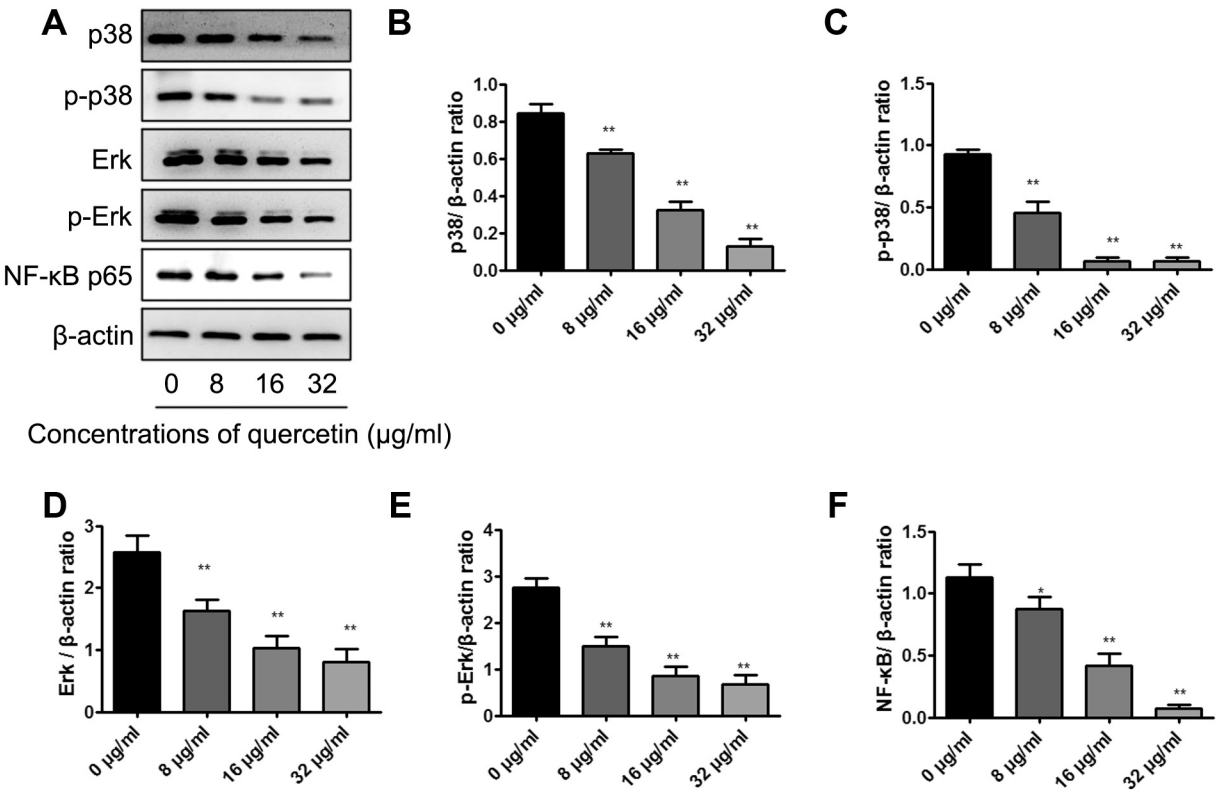

Fig. 4. Quercetin inhibits inflammation by down-regulating the p38MAPK and ERK1/2 and NF-κB pathways. J774 cells were infected with SS2 at different concentrations of quercetin (MOI = 8:1) for 5 h, and protein samples were analyzed by western blotting. Data are shown as the mean ± SD from more than 3 independent experiments. *$P < .05$ and **$P < .01$, by 2-tailed Student's *t*-tests.

SS2 activity and did not affect the viability of SS2. After SS2 was co-cultured with quercetin, the hemolytic activity of SS2 culture supernatants was evaluated by hemolysis assays. As shown in Fig. 1C, the bacterial culture supernatants without quercetin showed substantial lysis of red blood cells (69.32%). However, the hemolytic activity of the bacterial supernatants co-cultured with quercetin was significantly decreased in a dose-dependent manner (Fig. 1C). These findings indicate that this inhibition may be due to decreased production of SLY or decreased pore-forming activity of SLY induced by quercetin. The production of SLY in supernatants was not affected by quercetin treatment, as no difference was observed in the SLY protein band (Fig. 1D and E). Furthermore, consistent with the inhibition induced by quercetin for culture supernatants, the activity of purified SLY was significantly decreased in the presence of quercetin (Fig. 1F), suggesting that quercetin directly inhibits SLY pore-forming activity. Taken together, our findings demonstrated that quercetin treatment could directly neutralize SLY activity without affecting SS2 viability.

3.2. Quercetin inhibits the oligomerization of SLY

Oligomerization is critical for the activity for SLY. Thus, we hypothesized that quercetin treatment may affect the oligomerization of SLY and, subsequently, lead to the loss of hemolytic activity. As expected, the oligomerization of SLY was significantly reduced with the addition of quercetin (Fig. 2A and B). The monomers in the samples treated with quercetin were obviously increased and the oligomers significantly reduced compared to those in the sample without quercetin. Taken together, our results revealed that quercetin inhibited the pore-forming activity of SLY by suppressing the formation of oligomers.

3.3. Quercetin reduces the production of pro-inflammatory cytokines in SS2-infected cells

The pro-inflammatory cytokines in the supernatants of the co-culture system of J774 cells and SS2 were determined using ELSIA kits, and the results indicated that the production of the inflammatory mediators TNF-α, IL-6, and IL-1β was strongly activated in infected cells (Fig. 3). In contrast, the infected cells treated with quercetin showed significantly decreased production of TNF-α, IL-6, and IL-1β. Thus, the addition of quercetin in the co-culture system of J774 cells and SS2 strongly reduced the production of pro-inflammatory cytokines.

3.4. Quercetin decreases inflammation in SS2-infected cells by regulating the p38, ERK1/2 and NF-κB pathways

To further explore the anti-inflammatory mechanism of quercetin, we assessed the inflammation-related intracellular signaling pathways of p38, ERK1/2 and NF-κB in SS2-infected cells. The expression levels of p38 and ERK1/2 were significantly inhibited by the treatment with quercetin, which also attenuated the phosphorylation of p38 and ERK1/2. In addition, NF-κB, an important transcription factor that plays a key role in the regulation of pro-inflammatory cytokines, was detected after quercetin treatment. NF-κB expression in the infected cells treated with quercetin was strongly inhibited (Fig. 4E). Taken together, the results suggested that quercetin could inhibit the SS2-mediated inflammation by down-regulating the p38, ERK1/2 and NF-κB pathways.

3.5. Quercetin alleviates SS2-mediated cell damage

The LDH release in the supernatants of the co-cultured SS2 and J774 cells was further detected to verify the potential protective effect of quercetin on SS2 infection at the cellular level (Fig. 5D). The addition of

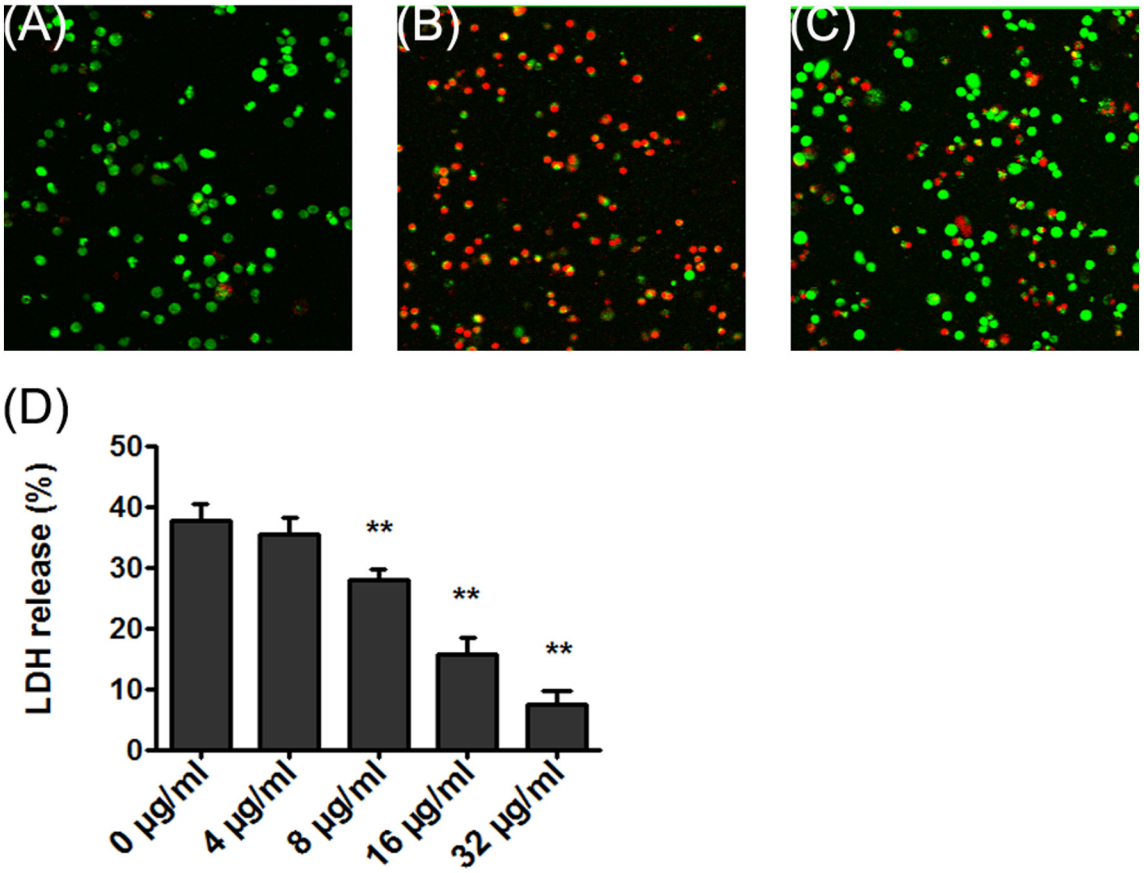

Fig. 5. Attenuation of SS2-mediatedcell damage by quercetin. A J774 cells were infected with SS2 for 5 h with or without quercetin and stained with live (green)/dead (red) reagent; the representative images were obtained by a confocal laser scanning microscope. B The release of LDH in J774 cells infected with SS2 for 5 h with or without quercetin was quantitatively measured by a Cytotoxicity Detection Kit (LDH). Data are shown as the mean ± SD from more than 3 independent experiments. $^{*}P < .05$ and $^{**}P < .01$, by 2-tailed Student's *t*-tests. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

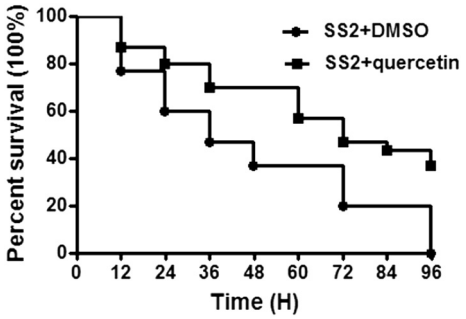

Fig. 6. Quercetin protects mice from SS2 infection. Mice infected with1×10^8 CFUs of SS2 were treated with quercetin or DMSO (negative control), and the mortality was observed for 96 h.

quercetin had no toxic effects on cells (Fig. 5A). The cell death was reduced in the presence of SS2; cell death decreased from 37.60% to 7.47% for the 32 μg/mL quercetin treatment vs the control without quercetin. (Fig. 5D) Most cells stained red (dead cells) in the sample infected with SS2 (Fig. 5B); however, the red cells were substantially decreased in the samples treated with quercetin (Fig. 5C). Taken together, our results suggested that quercetin inhibited the cell injury caused by SS2.

3.6. Quercetin protects mice from SS2 infection

A mouse model of SS2 infection was further employed to explore the protective role of quercetin in vivo. As shown in Fig. 6, the mortality of infected mice that received quercetin was significantly lower than that of mice treated with DMSO, which died in 4 days. Thus, quercetin treatment could reduce the mortality of SS2-infected mice.

4. Discussion

Similarly, anti-virulence strategies can also be used for SS2infections. Among the various virulence factors, SLY plays an important role in SS2 pathogenicity, as deletion of SLY can significantly reduce the pathogenicity of SS2 in mice [21]. In addition, SLY can increase the vascular permeability of mice, which is related to STSS [23]. Strains producing high-level SLY, but not strains with weak SLY secretion, are more likely to cause meningitis in humans [21]. In addition, SLY could facilitate *S. Suis* transport through the blood-brain barrier [19,44] in a mouse model of *S. suis* infection [21]. Thus, inhibiting the activity of SLY can be a potential approach to treat SS2 infection. In addition, the inflammatory response induced by *S. suis* infection is an important manifestation in the pathogenic process. For example, 50–60% of cases with clinical manifestations of *S. suis* infection were meningitis [45]. Thus, inhibition of inflammation is an important strategy in the response to *S. suis* infection.

Here, we discovered that quercetin, a natural compound widely found in plant products, could inhibit the pore-forming activity of SLY by suppressing the protein oligomerization without antibacterial activity, suggesting that this compound is a promising candidate for treatment of SS2 infection as anti-virulence agent. Although the bacterial viability was not affected, quercetin treatment significantly

alleviated the cellular injury caused by SS2 in J774 cells. Differs from the previous studies, quercetin showed potential inhibition of inflammatory responses mediated by SS2 in J774 cells, and the production of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, which play a crucial role in inflammatory diseases, was significantly decreased in the presence of quercetin. Additionally, by an in-depth evaluation of the anti-inflammatory effects of this compound, we found that quercetin could attenuate inflammatory responses by decreasing the members in MAPKs and NF-κB pathways, which are important in a variety of inflammatory responses.

Based on the above studies, our study, to the best of our knowledge, is the first to discover and elucidate the novel function of quercetin in two processes: anti-virulence (targeting SLY activity) and anti-inflammatory responses against SS2 infection. To improve and confirm the protective effect of quercetin against SS2 virulence, we performed tissue infection and mouse infection assays. As expected, the inhibitory effect of quercetin against SLY activity and inflammation could robustly protect host cells from SS2 infection with an attenuation of cytotoxicity for infected cells in the presence of quercetin. In the in vivo mouse model, quercetin treatment significantly enhanced the survival rate of infected mice. Thus, out results suggested that quercetin is a promising lead compound for the treatment of SS2 infection by targeting SLY activity and inflammatory responses, which can provide a theoretical basis for further development of new drugs.

Moreover, quercetin has a wide range of sources, which can reduce development costs to a certain extent, and this is also a major potential advantage for clinical R&D applications in the future. Although the mechanism of *S. suis* meningitis is still unclear, SLY and *S. suis* meningitis in humans are important. The dual effects of inhibition of SLY and inflammation by quercetin may play a role in the treatment of SS2-mediated meningitis, but the specific role needs to be further explored in future studies.

Conflicts of interest

None of the authors have a financial interest in any of the products, devices, or Materials mentioned in this manuscript. The authors declare that they have no conflicts of interest.

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