Astragalus polysaccharide strengthens the inflammatory and immune responses of *Brucella suis* S2-infected mice and macrophages

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Abstract. *Brucella* infection is one of the most serious zoonoses worldwide, affecting humans and domestic and wild animals. Astragalus polysaccharide (APS) is extracted from astragalus, which exhibits bioactive properties, including immunomodulation and anti-tumour and antiviral activity. The present study revealed that APS treatment promoted macrophage activation, the production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-12 and interferon-γ, and *Brucella* clearance in murine macrophages and spleens. APS treatment was also demonstrated to protect the integrity of macrophages during infection with live attenuated *Brucella suis* strain 2 (*B. suis* S2). The results from *in vitro* experiments were consistent with the findings from the *in vivo* study, showing the elevated secretion of TNF-α and nitric oxide in APS-treated murine peritoneal macrophages following *B. suis* S2 infection. The current study demonstrated the potential of APS in the control and treatment of *Brucella* infection, and the enhancement of host inflammatory and immune responses.

Introduction

Brucellosis is caused by the facultative intracellular pathogen *Brucella*, which is a gram-negative coccobacillus lacking capsules, flagella, endospores and native plasmids. This zoonotic disease affects the reproductive tract of livestock, resulting in abortion, infertility and consequently economic loss (1). Human infection is mainly due to the consumption of unpasteurized milk or undercooked meat from infected animals or from direct contact with the secretions of infected animals (1). Infection is characterized by undulant fever, physical weakness and the presence of diseases including endocarditis, arthritis and neurological disorders (2). Currently, a number of antibiotics can be used clinically for the treatment of human brucellosis, but no effective drugs have been identified for animal brucellosis (3). Live attenuated *Brucella* strains, including *B. suis* strain 2 (S2), are produced as vaccines to induce immunization against *Brucella* infection in domestic animals (4). The S2 vaccine is widely used in China as an effective method to prevent brucellosis in goats, sheep, cattle and swine, but it is not administered worldwide since its capacity to protect against heterologous virulent *Brucella* species is still debatable (5). Additionally, live attenuated vaccines are potential pathogens for humans and may induce abortion in pregnant animals (6). No safe or effective vaccines are currently available for the treatment of human brucellosis. Therefore, the identification of novel compounds for the prevention or treatment of brucellosis is urgently required.

*Brucella* species are relatively weak inducers of innate immunity and can eventually lead to a long-lasting infection (7). The replication of *Brucella* during infection is independent of toll-like receptor (TLR)4, TLR3, TLR2, TLR5 or the TIR-domain-containing adapter-inducing interferon-β adapter (TRIF), allowing the bacteria to evade immune detection and adapt to intracellular conditions (8-10). *Brucella* can evade phagocytic lysosomes and prevent phagosomal-lysosomal maturation to avoid degradation (11). *Brucella* has been indicated to prevent macrophages from secreting cytokines, undergoing apoptosis and presenting antigens to T cells (12). Macrophages are one of the primary targets during *Brucella* infection, accounting for the elimination of intracellular *Brucella* (13). Pro-inflammatory cytokines that are secreted by activated macrophages, including tumor necrosis factor (TNF)-α and interleukin (IL)-12, serve important roles in the regulation of early *Brucella* infection (3). TNF-α amplifies and mediates pro-inflammatory signalling pathways to enhance the host response upon *Brucella* infection (14). IL-12 can stimulate T helper 1 (Th1) and natural killer cells to participate in defence against *Brucella*, and the depletion of IL-12 has been revealed...
to impair the production of nitrous oxide (NO) and interferon (IFN-γ) (15). TNF-α and IL-12 are positive regulators of IFN-γ, which is the most potent inducer of anti-Brucella activity in monocytes and macrophages, and reduces the intracellular growth and replication of Brucella by stimulating the production of reactive oxygen species, reactive nitrogen species and TNF-α (16). Furthermore, cytokines can activate macrophages and protect hosts from prolonged infection (9).

Astragalus polysaccharide (APS) is a bioactive component that is extracted from astragalus. A study has identified APS as a bio-immunomodulator that promotes nonspecific and specific immunity, and as a vaccine adjuvant that enhances vaccine antigens (17). APS promotes the phagocytosis of macrophages, the proliferation of dendritic cell (DC) precursors and the ability of DCs to induce T cell proliferation and elevate the secretion of cytokines, including TNF-α, IL-6, IL-8, IFN-γ and IL-12 (18,19). The administration of APS to mice with sepsis can downregulate regulatory T cells (Treg) and restore the balance between Th1 and Th2 cells, leading to immunoregulation (20). Additionally, APS exhibits a number of additional biological activities, including anti-tumour, anti-oxidant and antiviral activity (21,22).

In the current study, the inflammatory and immune responses of mice and macrophages pretreated with APS were determined during Brucella infection. Enhanced cytokine production, macrophage activation and Brucella clearance were observed, and these observations demonstrated that APS improves the host response to Brucella in mice and is a promising candidate that can be used to control and prevent brucellosis.

Materials and methods

Preparation of APS. An APS injection (Qilu Animal Health Products Co., Ltd.) of 20 mg/ml was diluted with sterile normal saline solution and mixed prior to use.

Animals and administration. A total of 160 male BALB/c mice (age, 6-8 weeks) were supplied by Shanghai Laboratory Animal Centre. The animals were maintained in a specific pathogen-free (SPF) environment and kept under constant conditions, including 24±1°C temperature, 40-60% humidity and a 12 h light/dark cycle with access to food and water ad libitum. After acclimatization for one week, the mice were weighed and randomly divided into five groups. The infected control group was infected intraperitoneally with B. suis S2 (1x10⁴ CFU) treated with APS or bacteria. After co-incubation of APS (0, 50, 100 and 200 µg/ml) for 24 h treated with APS or bacteria. After co-incubation of APS (0, 50, 100 and 200 µg/ml) for 24 h and infected with B. suis S2 (1x10⁴ CFU live bacteria per animal) on day 4 via intraperitoneal injection. The other three groups (S2+0.3 mg/ml APS, S2+0.6 mg/ml APS and S2+1.2 mg/ml APS) were administered 0.3, 0.6 and 1.2 mg/ml APS by intraperitoneal injection once a day for 4 days, respectively, and infected intraperitoneally with B. suis S2 (1x10⁴ CFU per animal) on day 4. The mice were randomly selected for euthanasia at 1, 6, 24, 48 and 72 h after infection (n=6). Blood samples, peritoneal fluids and spleens were collected. An additional 10 mice were intraperitoneally injected with 2 ml 4% sterile starch solution, and sacrificed one day subsequent to the isolation of peritoneal macrophages.

Macrophage phagocytosis of bacteria and morphological examination of macrophages. The peritoneal fluids were collected from 1 and 72 h-infected groups (n=6). The phagocytic rate of macrophages was calculated using Wright-Giemsa staining (Sangon Biotech Co., Ltd.) according to the manufacturer's protocol. Macrophages were re-suspended in 0.5 ml phosphate buffer saline (PBS) and prepared as cell smears. After fixation in 95% ethanol at room temperature for 5 min, cells were stained with Wright-Giemsa for 8 min at room temperature and then washed with PBS. Cell samples were air-dried, and cell morphology was examined using a light microscope (magnification, x200). A total of 100 macrophages were counted manually, and the phagocytic rate was calculated using the following equation: Phagocytic rate = (the number of macrophages containing bacteria/100) x100%.

Determination of bacterial loads in macrophages and spleens. Peritoneal macrophages were collected at 1, 6 and 24 h from infected groups and spleens were collected at 6, 24 and 48 h from infected groups. A total of 2x10⁴ macrophages were lysed with 1 ml of 1% Triton X-100 (Beyotime Institute of Biotechnology) for 15 min. The lysate was diluted to 1:100 using sterile PBS. A total of 100 µl lysate was spread on TSA solid medium (Hangzhou Microbial Reagent Co., Ltd.), which was equivalent to the lysate of 20 macrophages, and then incubated for 72 h at 37°C. The spleens were weighed and homogenized with 1 ml of 1% Triton X-100. A total of 100 µl homogenate, which was equivalent to the homogenate of 10% of the whole spleen, was spread onto TSA solid medium and incubated for 72 h at 37°C. The numbers of bacteria in the macrophages and the spleens were determined by counting bacteria colonies. Log10 CFU/1,000 macrophages=Log10 (colony count x500). Log10 CFU per gram spleen=Log10 (colony count x10)/spleen weight (g).

Peritoneal macrophage culture. Peritoneal macrophages were isolated from BALB/c mice 1 day after intraperitoneal injection of 2 ml 4% sterile starch solution. A total of 2x10⁶ cells/ml were suspended in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 4 mM L-glutamine (Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), incubated in 12 or 96-well culture plates in a humidified 5% CO₂ incubator at 37°C for further use.

Measurement of cytokines and NO production. The levels of TNF-α, IL-12 and IFN-γ in mouse serum collected at 6, 24 and 48 h time points were measured using ELISA kits (NeoBioscience Technology Co., Ltd.). The serum was obtained by centrifugation at 1200 x g, room temperature for 10 min. For the in vitro assay, peritoneal macrophages were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 4 mM L-glutamine (Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in 12-well plates in the presence of different concentrations of APS (0, 50, 100 and 200 µg/ml) for 24 h in a humidified 5% CO₂ incubator at 37°C. B. suis S2 was then loaded at a multiplicity of infection, at 100:1. The uninfected group was not treated with APS or bacteria. After co-incubation in a humidified...
5% CO\textsubscript{2} incubator at 37°C for 1 h, extracellular bacteria were washed away by removal of the medium and washing with PBS, and previous concentrations of APS (0, 50, 100 and 200 µg/ml) in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 4 mM L-glutamine (Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) were added. The cells then were incubated in a humidified 5% CO\textsubscript{2} incubator at 37°C for 24 h. The levels of NO and TNF-\alpha in the culture supernatant were determined using a total nitric oxide assay kit (Beyotime Institute of Biotechnology) and an ELISA kit (NeoBioscience Technology Co., Ltd.), respectively, at 24 h after the medium was replaced.

Cell viability. Murine peritoneal macrophages were seeded at a density of 2x10\textsuperscript{5} cells/well in a 96-well plate. After attachment, the cells were treated with the indicated concentrations of APS (0, 25, 50, 100, 200 or 400 µg/ml) and incubated in a humidified 5% CO\textsubscript{2} incubator at 37°C for 48 h. Cell viability was analysed using MTT as previously described (23). Briefly, MTT solution was added to the cells at a final concentration of 0.5 mg/ml for 4 h at 37°C in a humidified 5% CO\textsubscript{2} atmosphere. The medium was then removed and 150 µl of DMSO was added to each well. The optical density of each well was measured at 570 nm.

Statistical analysis. The data were expressed as the mean ± SEM or mean ± standard deviation. A one-way ANOVA followed by a Tukey's or Dunnett's test was performed using GraphPad Prism 5 software (GraphPad Software, Inc.) to determine statistical comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of APS on the morphology of macrophages from B. suis S2-infected mice. Wright-Giemsa staining was used to examine macrophage morphology. After the mice were infected with B. suis S2 for 1 or 72 h, peritoneal macrophages were collected and stained. As presented in Fig. 1A, at 1 h after infection, the macrophages from the APS-treated groups ingested more bacteria than those from the Brucella-infected control group. In the 72 h-infected samples, bacteria were identified at very low levels inside the macrophages from each group (Fig. 1B). The membrane integrity of macrophages from the S2-infected group was destroyed, as the vacuoles were formed in the cytoplasm and the nucleuses were broken (Fig. 1B). In the groups treated with 0.6 and 1.2 mg/ml APS respectively and infected with B. suis S2 for 72 h, the membranes and the nuclei were intact, and the cytoplasm was normal (Fig. 1B). In the S2+0.3 mg/ml APS group, the membranes and nuclei remained intact with prominent nucleoli, but the size of the macrophage increased and the vacuoles appeared around the nucleus in the cytoplasm (Fig. 1B). These results demonstrated that APS promoted macrophage activation and protected the integrity of macrophages during infection.

Effects of APS on macrophage phagocytosis of B. suis S2 and bacterial loads in macrophages. To assess the phagocytosis of macrophages, the phagocytic rate, which is the percentage of macrophages ingesting Brucella, was measured at 1 h after infection. The phagocytic rates of macrophages were increased in the groups pretreated with APS compared with the mice infected with Brucella alone, implying that APS might stimulate macrophages to ingest bacteria (Fig. 2A). The bacterial loads in peritoneal macrophages from each group were examined at 1, 6 and 24 h following infection. The amount of Brucella engulfed by macrophages was relatively high at 1 h after infection, and as time progressed, the load markedly declined (Fig. 2B). Compared with the macrophages from the B. suis S2-infected mice without APS protection, the macrophages from the 1.2 mg/ml APS-administered mice engulfed significantly more Brucella at the 1 h time point, and no Brucella remained inside the macrophages at 24 h following infection (Fig. 2B). Furthermore, a dose-dependent decline in the number of intracellular bacteria was observed at the 6 and 24 h time points (Fig. 2B). In conclusion, these results suggested that APS was able to promote macrophage phagocytosis of B. suis S2 in a dose-dependent manner and accelerate the removal of Brucella to avoid long-term infection.

APS elevated the levels of serum TNF-\alpha, IL-12 and IFN-\gamma during B. suis S2 infection. Cytokines are critical effector molecules in the control of Brucella growth and brucellosis treatment, and the level of cytokines is associated with the intensity of the host immune response (24). Consequently, cytokine content in the serum from mice in each group was analysed at the 6, 24 and 48 h time points. Compared with the uninfected control, the B. suis S2-infected mice exhibited enhanced production of TNF-\alpha, IL-12 and IFN-\gamma at all the time points, and in the APS-treated S2-infected groups, the levels of all the tested cytokines were higher than those in the S2-infected control group, except for the IL-12 levels at 6 h post-infection (Fig. 3A-C). At 24 h post-infection, the secretion of TNF-\alpha, IL-12 and IFN-\gamma in the S2+1.2 mg/ml APS group increased by 3.25-, 2.93- and 3.62-fold, respectively, compared with that of the S2-infected control group (Fig. 3A-C). IL-12 levels remained similar in the S2-infected control group as the infection time increased, while IL-12 levels in the APS-treated S2-infected mice was higher at the 24 h time point compared with the 6 h time point (Fig. 3B). IFN-\gamma levels in the S2-infected control group increased modestly in a time-dependent manner during 48 h infection and decreased in the 0.6 and 1.2 mg/ml APS-treated S2-infected mice (Fig. 3C). However, the serum concentration of IFN-\gamma was higher in the APS-administered mice at all the time points (Fig. 3C). In conclusion, APS treatment can induce cytokine production, facilitating the inflammatory reaction and immunological response against Brucella infection.

Bacterial loads in the spleen were affected by APS. The spleen is a major organ that serves an important role in the immune response and contains a high number of bacteria during brucellosis (25). Therefore, spleens were collected from B. suis S2-infected mice at 6, 24 and 48 h post-infection and the number of bacteria in each spleen was determined. The average weight of spleens from each infected group collected at 24 h post-infection showed no significant difference (Fig. 4A). The bacterial loads in the spleen were increased in the S2-infected control mice as the infection time increased, and in contrast,
the maximum CFU detected in the spleen from the 0.6 and 1.2 mg/ml APS-treated S2-infected mice was at 6 h after infection (Fig. 4B). At 6 h post-infection, the number of spleen bacteria in the 1.2 mg/ml APS-treated S2-infected mice was significantly higher than that in the S2-infected control mice, and at 48 h after infection, the results were reversed (Fig. 4B). The results revealed that APS had a positive effect on the immune response in mice and improved the clearance of Brucella in the spleen.

Enhanced production of TNF-α and NO by APS following Brucella infection in vitro. A cell viability assay was performed to determine the toxicity of APS and the suitable dose for in vitro assay. The tested concentrations of APS exhibited no significant influence on the viability of murine peritoneal macrophages (Fig. 5A). For the cytokine assay, 200 µg/ml APS was selected as the highest concentration. After attachment, peritoneal macrophages were treated with 0, 50, 100 or 200 µg/ml APS for 24 h and infected with B. suis S2 for 1 h. After infection, the original medium was replaced with fresh medium containing the same concentrations of APS that were present previously. A period of 24 h later, the culture supernatant was collected, and the content was detected. Consistent with the in vivo results, the level of TNF-α in the cell culture medium was significantly increased by APS after infection, indicating the promotion of macrophage activation (Fig. 5B). NO production by activated macrophages has been indicated to mediate the intracellular replication and killing of Brucella (26). As illustrated in Fig. 5C, APS-induced macrophages were observed to produce a relatively large amount...
of NO that may have suppressed the survival of intracellular Brucella. In summary, APS facilitated the production of the pro-inflammatory effectors TNF-α and NO to eradicate Brucella.

Discussion

The aim of the current study was to examine the potential effect of APS on the protection and treatment of Brucella infection. Due to lab safety issues, the B. suis S2 vaccine, which is of low virulence, was used instead of virulent Brucella. The results demonstrated that APS treatment in mice promoted the secretion of pro-inflammatory cytokines and macrophage activation during Brucella infection to enhance the inflammatory and immune responses of the host, and ultimately accelerated Brucella clearance in macrophages and spleens to avoid long-term infection. The induction of TNF-α and NO by APS was observed in in vitro experiments. The present study demonstrated that the protective role of APS in Brucella infection is at least partially due to the enhancement of host defence reactions.
The mouse model is widely used to study the interaction between Brucella and the immune system due to the practicality of this model and the avoidance of economic loss and ethical issues. Innate immune and adaptive immune systems participate in the resistance to brucellosis. During infection, macrophages are the first line of defence in the innate immune response. The current study indicated that at the early stages of infection, the phagocytic activity of macrophages was intensified in the APS-treated groups, and at the late stages of infection, the APS-treated mice had a decreased number of bacteria inside the peritoneal macrophages compared with the infected control group, suggesting that APS promotes the killing of Brucella via macrophage activation. Consistently, a rapid clearance of Brucella was observed in the spleens of the APS-treated mice, while in the infected control group, the bacterial loads in spleens were increased as infection time increased, indicating that a number of bacteria evaded the bactericidal function of phagocytic cells and had successfully survived and multiplied inside the cells. Typically, within the first few hours after infection, the majority of bacteria are eliminated by macrophages (27). However, some pathogens may survive due to the Brucella species exhibiting strategies to evade innate immunity. The mechanism of evasion includes the inhibition of TLR signalling and suppression of the complement system and is associated with the specific structures of Brucella species, including lipid A, modified LPS with longer fatty acid residue and flagellin (28).

TNF-α, IL-12 and IFN-γ are cytokines that initiate host defence responses and modulate the activity of immune-associated cells in brucellosis (3,14,24). In the present study, the serum levels of TNF-α, IL-12 and IFN-γ were determined in mice to assess the production of cytokines from spleen cells (3). APS treatment enhanced the secretion of TNF-α, IL-12 and IFN-γ during infection, indicating a stronger potential for macrophage activation and bactericidal function. In support of in vitro research, the current in vitro study using mouse peritoneal macrophages demonstrated that APS increased the production of TNF-α and NO following Brucella infection, suggesting that APS suppressed bacterial intracellular replication. Pro-inflammatory cytokines, including TNF-α and IFN-γ can induce the killing ability of macrophages, which is responsible for Brucella elimination in infected mice (14,24). The failed induction of TNF-α is associated with reduced toxicity of host cells and favours the replication of intracellular Brucella (29). An in vitro study has demonstrated that macrophages activated by TNF-α can inhibit Brucella replication (30). IFN-γ is produced by CD4+, CD8+ and T lymphocytes and serves an important role in the clearance of Brucella. IFN-γ induces macrophages to degrade intracellular bacteria and CD8+ and T cells to exert cytotoxic effects on infected macrophages (3). Furthermore, IFN-γ mediates the type I immune response and prompts the expression of MHC-I and MHC-II against brucellosis (28,31). Brucella species exhibit the ability to repress the secretion of the aforementioned cytokines to interfere with the activity of phagocytic cells and the protective Th1 immune response (32). Following Brucella invasion and internalization, the production of pro-inflammatory cytokines is often reduced due to TLR inhibition, incomplete antigen presentation and the inhibition of DCs and naïve T cell maturation (32,33).

The present study demonstrated that APS enhanced host defences during Brucella infection, and provide novel insight into the use of APS in vaccine immunization. Zhou et al reported that APS increases NO, TNF-α, IL-1β and IL-6 concentration through TLR4- and MyD88-dependent pathways to modulate host immunity (34). As a vaccine adjuvant, APS has been indicated to improve the immune response against H5N1 avian influenza virus (AIV) and reduce the replication of H9N2 subtype AIV in chickens (35,36). Additionally, APS has been revealed to alleviate ochratoxin A-induced immune stress by reducing pro-inflammatory cytokine expression, cell apoptosis and spleen damage (37). A mechanism study indicated that APS activates the AMPK/SIRT-1 pathway and inhibits NF-κB against immune stress (37). Previous studies have indicated that APS can modulate the immune response, and can ameliorate over-reactive immune stress and boost the host immune response during pathogen invasion, providing balance in the immune system (34,36).

In conclusion, the current study highlights the potential use of APS for the control of Brucella infection. Future studies should determine whether APS can improve the protective efficacy of the S2 vaccine and its underlying mechanisms to further verify its therapeutic efficacy and safety when used against virulent Brucella.
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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors’ contributions

QS performed the experiments and wrote the manuscript. L. Zhao collected and analyzed the data. L. Zhang designed and performed the experiments. All authors read and approved the final manuscript.

Ethical approval and consent to participate

The experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang Ocean University and adhered to the code of the World Medical Association (Declaration of Helsinki).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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