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Original Article

Outer membrane protein 31 of *Brucella* promotes apoptosis of astrocytes via the p38MAPK/MK2 signaling pathway

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Abstract: Neurobrucellosis is an inflammatory disorder caused by infection of *Brucella* in central nervous system (CNS). The *Brucella* infection induced the secretion of cytokines in astrocytes, such as TNF-α, IL-6, and IL-1β, which played an important role in the immunophathogenesis of neurobrucellosis. The outer-membrane protein 31 (Opm31) in *Brucella melitensis* functions as an immunodominant and protective antigen during the course of *Brucella* infection. The purpose of this study was to investigate the potential pathogenic mechanism of neurobrucellosis induced by *Brucella* in astrocytes. ELISA assay showed that the concentrations of TNF-α, IL-6, IL-1β, and IL-10 in astrocytes in Opm31 groups were significantly increased compared with control group. Flow cytometry with annexin V-FITC/PI staining indicated that Opm31 protein significantly promoted the apoptosis of astrocytes *via the mitochondrial pathway.* Moreover, Opm31 significantly increased the phosphorylations of p38MAPK and MK2. Si-p38MAPK and p38MAPK inhibitor SB203580 significantly decreased Opm31-induced apoptotic rates. Opm31 of *Brucella* promoted the apoptosis of astrocytes via the p38MAPK/MK2 signaling pathway, providing a possible pathogenesis of neurobrucellosis infected by *Brucella* in astrocytes.

Keywords: Apoptosis, astrocytes, *Brucella*, outer membrane protein 31, p38MAPK/MK2

Introduction

Brucellosis caused by *Brucellae* is a widespread infectious and zoonotic disease that affecting humans and animals, and it is characterized by undulant fever, arthritis, abortion and infertility in a variety of animals [1]. Brucellosis is an inflammatory disease and has caused dramatic economic losses for livestock industry, agriculture and public health, especially in developing countries. Though great efforts have been made to develop effective vaccines against brucellosis, there is still no satisfactory progress to defend this disease in human [2]. Neurobrucellosis is a crucial complication of systemic brucellosis infection in the central nervous system (CNS) and could cause inflammatory disorders and tissue destruction, such as meningitis, meningoencephalitis and polyradiculitis [3]. Thus, it is extremely helpful to understand the potential pathogenesis of neurobrucellosis to improve its therapeutic effect.

Innate immune in the CNS is essential to detect and clear bacterial pathogens and is regularly activated by various pathways when recognizing invasive pathogens [4]. Astrocytes, the most numerous cell type in the CNS, play an important role in maintaining innate immunity, blood-brain barrier (BBB) integrity, and function of CNS [5]. The infection of astrocytes caused the secretion of cytokines TNF-α, IL-6, and IL-1β and chemokines such as CCL2 and CXCL1 [6].

Brucellae, with an extensive host range, are Gram-negative, facultative intracellular and coccobacillus bacteria which belong to the alpha subclass of Proteobacteria. *Brucellae* lacks classical virulence factors, such as invasive proteases, virulence plasmids and lysogenic phages, and instead expresses virulence factors associated with the capacity to invade and propagate within host cells [7]. *Brucellae* triggered multiple system damages in human and

animals, among which the CNS injury approximately accounted for 1.7%-10% [8].

The major outer membrane proteins (Omps) of *Brucella* are mainly divided into two groups: group 2 (Omp2a and Omp2b) and group 3 (Omp25 and Omp31). Omp31 is found in almost all *Brucella* species, except for *Brucella abortus* [9]. Opm31 protein was able to form oligomers resistant to denaturation by sodium dodecyl sulfare (SDS) at low temperatures in *B. melitensis* [10]. *Omp31* gene was initially cloned and sequenced from *B. melitensis* 16 M and shared 34% identity with *Opm25* [10]. It is reported that group 3 proteins, especially Omp31, functions as an immunodominant and protective antigen of *B. ovis* infection in rams [10]. Gupta *et al*. reported that vaccines of recombinant invasive *Escherichia coli* expressing *B. melitensis* Opm31 provided the protection for mice against virulent *B. melitensis* and promoted cellular immune responses [11]. However, the relationship between Opm31 and the damage caused by *Brucella* remains unclear.

p38 mitogen-activated protein kinase (p38-MAPK) is a common intracellular signaling transduction pathway which is always activated by phosphorylation of p38MAPK [12]. MAPKAP kinase 2 (MK2), a protein kinase activating the downstream of p38MAPK, is a main regulator of stress- and cytokine-induced post-transcriptional gene expression [13, 14]. The p38MAPK signaling pathway is closely related to the regulation of neuronal growth, proliferation, apoptosis, and pro-inflammatory cytokine secretion [15, 16]. Experimental evidences have showed that the activation of p38MAPK is strongly associated with astrocyte senescence-related secretory phenotype [17].

In this study, the effect of Opm31 on apoptosis of astrocytes was investigated and the potential relationships between Opm31 and the p38MAPK/MK2 signaling pathway was further studied in astrocytes.

Materials and methods

Primary astrocytes culture

Human astrocytes of cortical origin (ScienCell, San Diego, CA USA) were used for the primary cultures of astrocytes as previously described [18]. Briefly, human astrocytes of cortical origin were used to dissociate and produce single cell suspension by enzymatic hydrolysis and then the cell suspension was cultured on negative immunopanning plates to isolate astrocytes. The isolated astrocytes were grown in a Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Hyclone, Logan, Utah, USA) containing 10% fetal calf serum (Invitrogen, Grand Island, NY, USA), Nutrient Mixture F-12, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine at 37°C in humidified 5% CO_2 and 95% air. When the cells were grown to 80% confluency, the culture media of astrocytes were replaced with serum-free media.

Cell transfection

Small interfering RNA against p38MAPK (si-p38MAPK) and corresponding negative control (si-control) were purchased from GenePharma Biotech (Shanghai, China). Cells were plated into six-well plates and cultured for 24 h, and then 100 pmol/L of these molecular products were transfected into astrocytes by Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA).

Purification of recombinant Omp31 expression

The primer of Opm31 gene was designed and synthesized depending on the nucleotide sequences of Brucella outer membrane protein gene in GenBank. The *Omp31* gene was then amplified with Opm31 primers by polymerase chain reaction (PCR) and cloned into *Not*I and *Nde*I sites of PET-30a plasmid to construct the recombinant plasmid PET-30a-Opm31. Subsequently, the recombinant plasmid was transformed into competent cells *Escherichia coli* BL21 (DE3) to overexpress recombinant proteins Opm31 induced by 0.1 mM IPTG. The recombinant proteins were purified by affinity chromatography with Ni (II)-conjugated Sepharose at 4°C and quantified by BCA protein assay kits (Bio-Rad Laboratories, Inc. Hercules, CA, USA). SDS-polyacrylamide gel (SDS-PAGE) was performed to confirm the expression of Opm31.

Cytokine detection by ELISA assays

Astrocytes were seeded in 6-well plates at a density of 1×10^6 cells/ml and incubated in DMEM-HG medium at an incubator of 5% CO_2

at 37°C for 2 h. Then the cells were collected, washed three times and resuspended in DMEM-HG medium treated with different concentrations (0, 25 and 50 ng/ml) of Opm31. After incubation for 12 h, 24 h, and 48 h, the concentrations of cytokines (TNF-α, IL-6, IL-1β, and IL-10) produced in culture supernatant of each sample were detected based on the standard curves of known concentrations of recombinant cytokines by ELISA antibody kits (AlerChek, Portland, Maine, USA) following the manufacturer's protocol.

Western blot analysis

Astrocytes were seeded into 6-well plates and treated with different concentrations (0, 25 and 50 ng/ml) of Opm31 for 24 h and 48 h in a 5% CO_2 incubator at 37°C. After incubation for 48 h, cells were collected and lysed by ice-cold RIPA buffer (Zhong-Shan Jinqiao, Beijing, China) for 30 min. The extracted protein concentrations were determined by BCA protein assay kits (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Equal amounts of prepared protein samples (50 mg per sample) were mixed with 5 × loading buffer, denatured at 100°C for 5 min in a water bath and then analyzed by 10% SDS-polyacrylamide gels (SDS-PAGE) electrophoresis. After electrophoresis, the protein samples were transferred to polyvinylidene fluoride (PVDF; Millipore, Bedford, MA, USA) and then blocked with 5% non-fat milk in TBST at room temperature. The membrane was washed three times and probed with rabbit anti-Opm31, rabbit anti-Bcl-2, rabbit anti-Bax, rabbit anti-Cyt-C, rabbit anti-total cascase-3, rabbit anti-phosphorylated caspase-3, mouse anti-phosphorylated p38MAPK, mouse anti-phosphorylated MK2 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-β-actin (1:2500 dilution; Santa Cruz Biotechnology) on a rocking platform overnight at 4°C. Then the membrane was washed three times with TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution; Santa Cruz Biotechnology) for 2 h at room temperature. The protein bands were visualized by using enhanced chemiluminescence detection reagent (Super-Signal West Pico Chemiluminescent Substrate; Thermo Scientific, Waltham, MA, USA) and normalized to β-actin.

Assessment of apoptosis

Astrocytes (1 × 10^5 cells/ml) or astrocyte cells with si-p38MAPK or si-control were treated with different concentrations of Opm31 (0, 25 and 50 ng/ml) for 24 h and 48 h, or SB203580 (0, 0.05 and 0.1 μM) for 48 h. The treated cells were then stained with FITC conjugated Annexin V and propidium iodide (PI) using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology, Nantong, China) as described previously [19, 20]. The stained samples were analyzed by a flow cytometer (FACSCanto; BD Bioscience, San Jose, CA, USA) to evaluate the percentage of apoptotic cells.

Statistical analysis

All data were showed as mean ± standard deviation (SD) and analyzed using the GraphPad Prism Software version 5. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). A *P* value <0.05 was considered to be statistically significant.

Results

Opm31 promoted production of cytokines in astrocytes

The recombinant protein Opm31 was purified (one signal band on SDS-PAGE) and the concentration of purified Opm31 proteins by Ni-NTA affinity column was approximately 0.23 mg/ml. To investigate the effect of Opm31 on the expression of cytokines including IL-6, TNF-α, IL-1β, and IL-10, astrocytes were treated with different concentrations (0, 25 and 50 ng/ml) of Opm31 for 12 h, 24 h, and 48 h. The cells treated with PBS were used as control. The concentrations of IL-6, TNF-α, L-1β, and IL-10 in the supernatant of astrocytes were detected by ELISA Assays. As shown in **Figure 1A-D**, the concentrations of IL-6, TNF-α, IL-1β, and IL-10 in Opm31 groups were significantly increased in a time-dependent manner compared with PBS control group. In addition, the concentrations of IL-6, TNF-α, IL-1β, and IL-10 in 50 ng/ml Opm31 group were all significantly higher than that in 25 ng/ml Opm31 group. These data suggested that *Opm31 induced the section of cytokines IL-6, TNF-α, IL-1β, and IL-10 in astrocytes.*

Opm31 promoted apoptosis of astrocytes

After astrocytes were treated with different concentrations (0, 25 and 50 ng/ml) of Opm31

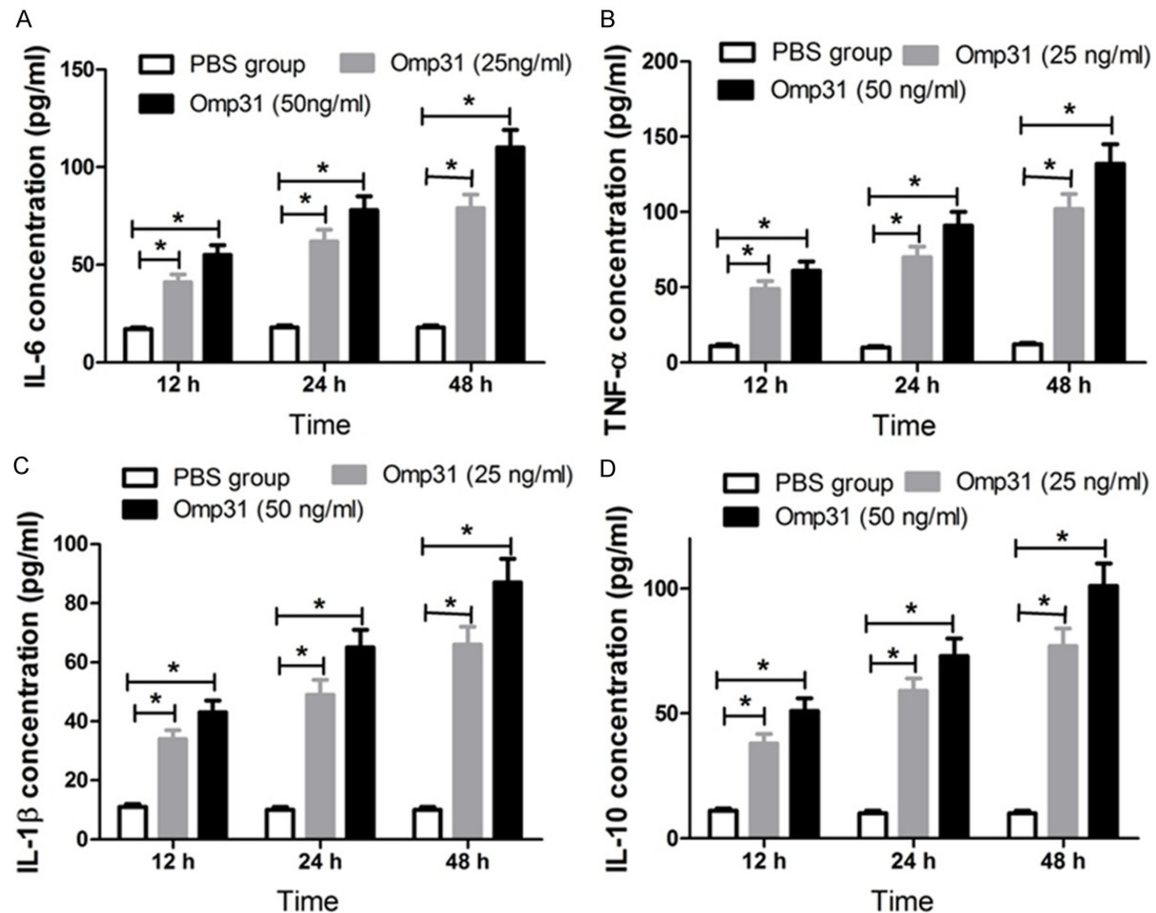

Figure 1. Opm31 protein promoted the production of cytokines in astrocytes. A-D: The concentrations of IL-6, TNF-α, IL-1β and IL-10 in astrocytes treated with different concentrations (0, 25 and 50 ng/ml) of Opm31 were all detected by ELISA Assays. Data are presented as the mean ± SD. *P<0.05.

for 24 h and 48 h, apoptosis was assessed by Annexin V-FITC double staining and flow cytometry analysis. The results indicated that the apoptosis rates were significantly increased both in 25 ng/ml and 50 ng/ml Opm31 groups at 24 h (**Figure 2A-D**) and 48 h (**Figure 2E-H**) compared with PBS control group. Besides, the apoptosis rates in 50 ng/ml Opm31 group were significantly higher than that in 25 ng/ml Opm31 group and the apoptosis rates at 48 h were also higher than that at 24 h. These results indicated that Opm31 increased apoptosis of astrocytes.

Opm31 induced apoptosis of astrocytes via the mitochondrial pathway

To further verify the effect of Opm31 on the apoptosis of astrocytes, the levels of apoptotic-related proteins Bcl-2, Bax, Cyt-C and caspase-3 were detected by western blot. The results showed that the Bcl-2 expressions were significantly decreased and Bax levels were significantly increased both in 25 ng/ml and 50 ng/ml Opm31 groups at 24 h (**Figure 3A**) and 48 h (**Figure 3B**) compared with PBS control group. In addition, Bcl-2 levels in 50 ng/ml Opm31 group were significantly lower than that in 25 ng/ml Opm31 group and Bax levels were significantly higher than that in 25 ng/ml Opm31 group (P<0.05). The level of Cyt-C and p-caspase-3 were significantly increased both in 25 ng/ml and 50 ng/ml Opm31 groups at 24 h (**Figure 3C**) and 48 h (**Figure 3D**) compared with PBS control group (P<0.05). Furthermore, the levels of Cyt-C and p-caspase-3 in 50 ng/ml Opm31 group were significantly higher than that in 25 ng/ml Opm31 group. All the results demonstrated that the *Opm31 promoted apoptosis of astrocytes via the mitochondrial pathway.*

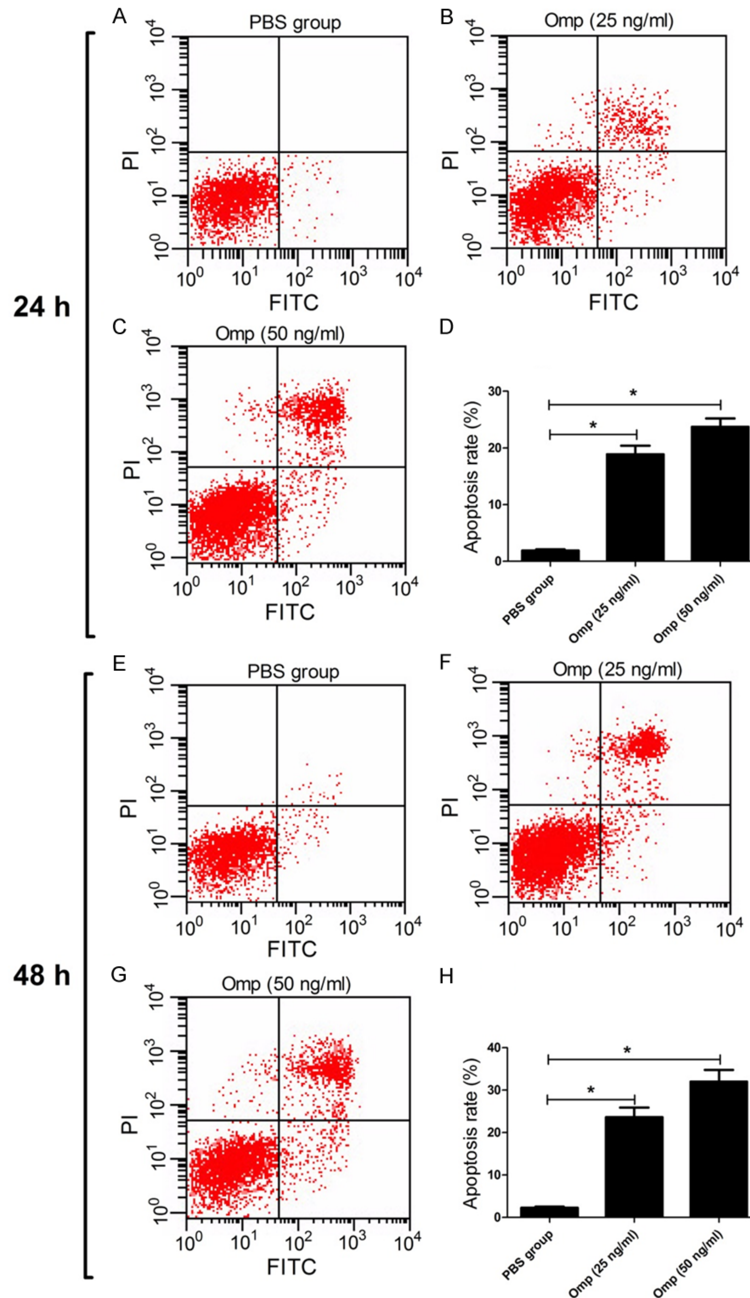

Figure 2. Opm31 protein promoted the apoptosis of astrocytes. A-D: Apoptosis rates of astrocytes treated with different concentrations (0, 25 and 50 ng/ml) of Opm31 at 24 h were assessed by flow cytometry. E-H: Apoptosis rates of astrocytes treated with different concentrations (0, 25 ng/ml, and 50 ng/ml) of Opm31 at 48 h were assessed by flow cytometry. Each group was performed in three independent experiments. Data are presented as mean ± SD. *$P<0.05$.

Opm31 protein induced cell apoptosis via the p38MAPK/MK2 signaling pathway in astrocytes

Since the p38MAPK/MK2 signaling pathway is closely related to the regulation of neuronal cellular growth, apoptosis, inflammatory responses and pro-inflammatory cytokine secretion which is promoted by Opm31, the relationship between Opm31 and the p38-MAPK signaling pathway was investigated in astrocytes. After astrocytes were treated with different concentrations (0, 25 ng/ml, and 50 ng/ml) of Opm31, western blot was used to detect the phosphorylations of p38MAPK and MK2. The results revealed that Opm31 significantly increased the phosphorylations of p38MAPK and MK2 in 25 ng/ml and 50 ng/ml Opm31 groups compared with PBS control group (**Figure 4A** and **4B**). Besides, phosphorylations of p38MAPK and MK2 in 50 ng/ml Opm31 group were significantly higher than that in 25 ng/ml Opm31 group.

Si-p38MAPK and si-control were used to further explore the role of the p38MAPK/MK2 signaling pathway in astrocytes apoptosis induced by Opm31. Apoptosis of astrocytes with 50 ng/ml Opm31 treatment and si-p38MAPK or si-control transfection was assessed by flow cytometry. The results indicated that the apoptosis rates of astrocytes with 50 ng/ml Opm31 treatment and si-p38MAPK transfection were significantly lower than that of astrocytes with 50 ng/ml Opm31 treatment and si-control transfection (**Figure 4C**).

SB203580, a pyridinyl imidazole inhibitor of p38MAPK, was added into astrocytes in combination with 50 ng/ml Opm31. The results showed that the apoptosis rates of astrocyte cells in 50 ng/ml Opm31 + SB203580 group were significantly decreased compared with

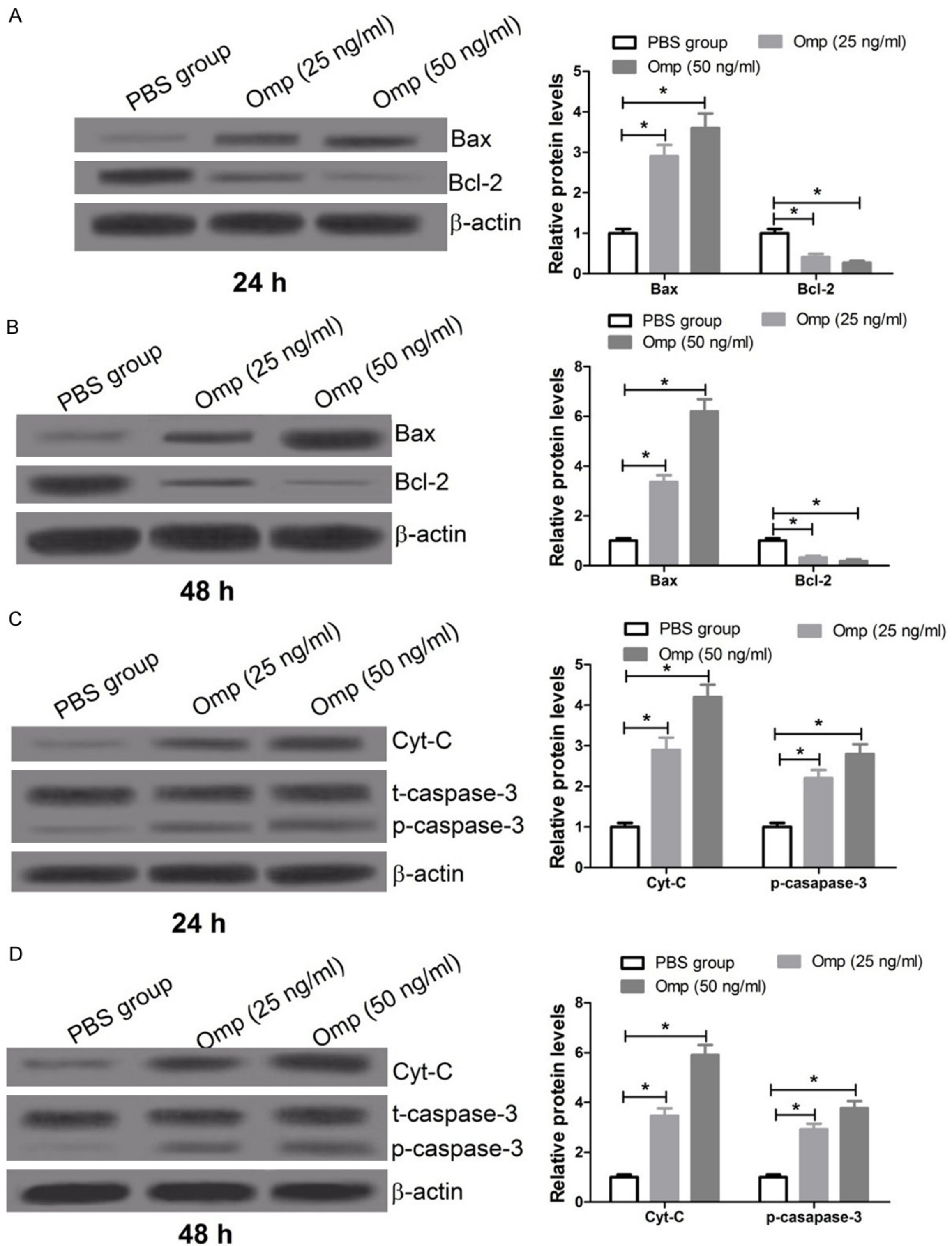

Figure 3. Opm31 promoted the apoptosis of astrocytes via the mitochondrial pathway. The levels of Bcl-2 and Bax in astrocytes treated with different concentrations (0, 25 ng/ml, and 50 ng/ml) of Opm31 at 24 h (A) and 48 h (B) were detected by western blot. Cyt-C levels and phosphorylation of caspase-3 were evaluated by western blot after astrocytes were treated with different concentrations (0, 25 ng/ml, and 50 ng/ml) of Opm31 at 24 h (C) and 48 h (D). Data are presented as mean ± SD. *P<0.05.

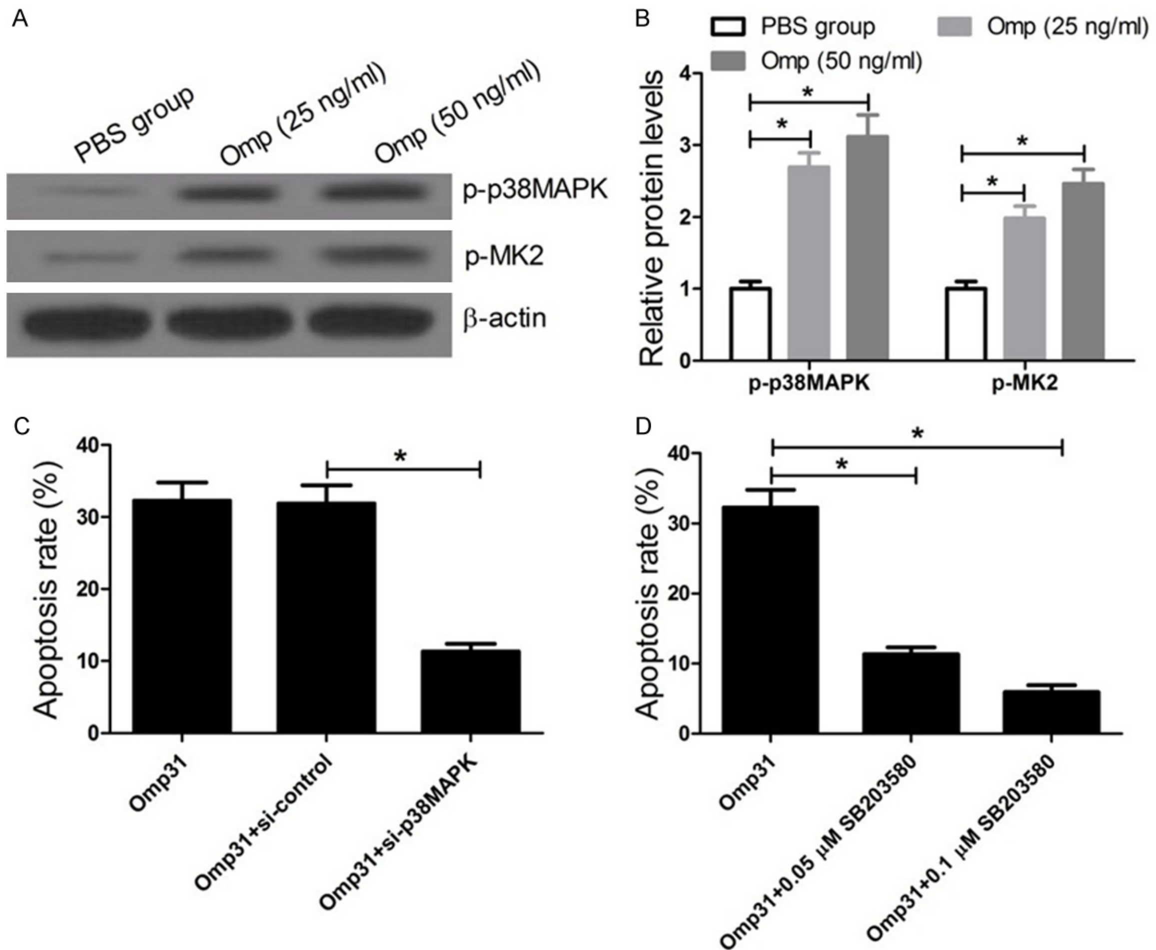

Figure 4. Opm31 protein suppressed cell apoptosis via p38MAPK/MK2 signal pathway. A: The phosphorylations of p38MAPK and MK2 in astrocytes treated with different concentrations (0, 25 ng/ml, and 50 ng/ml) of Opm31 were evaluated by western blot. B: The quantitative analysis of p-p38MAPK and p-MK2 in astrocytes treated with different concentrations (0, 25 ng/ml, and 50 ng/ml) of Opm31 were displayed and normalized to β-actin. C: Apoptosis of astrocytes with 50 ng/ml Opm31 treatment and si-p38MAPK or si-control transfection was assessed by flow cytometry. D: Apoptosis of astrocytes with 50 ng/ml Opm31 treatment together with 0.05 μM or 0.1 μM SB203580 was measured by flow cytometry. Data are presented as mean ± SD. *P<0.05.

that in 50 ng/ml Opm31 group (**Figure 4D**). Moreover, treatment of 0.1 μM SB203580 led to a lower apoptosis rates of astrocytes than 0.05 μM SB203580. Together, these data demonstrated that inactivation of p38MAPK/MK2 pathway attenuated Omp31-induced apoptosis in astrocytes.

Discussion

Brucella strains, including *Brucella melitensis* can result in numerous complications of affected organs. The incidence of brucellosis relapse still remains high (5%-40%) even with the appropriate therapeutics treatment [21]. Neurobrucellosis is a rare but serious form of brucellosis in CNS. Therefore, brucellosis including neurobrucellosis is still an important public health problem all over the world [22]. However, the pathogenic mechanisms of *Brucellae* in astrocytes are still obscure. Pro-inflammatory mediators of cytokines including IL-6, TNF-α, IL-1β and IL-10 are crucial regulators for inflammatory diseases. For example, TNF-α is involved in cell proliferation, apoptosis and inflammatory responses in astrocytes [23]. IL-6 promotes the apoptosis of astrocytes and is associated with bacterial and viral infections [24]. IL-10 can promote innate immune responses to reduce the damage caused by bacterial and viral infection [25]. Additionally, IL-1β palys a crucial role in the regulation of

BBB permeability, inflammatory activation of resident glia and human brain microvascular endothelial cells (HBMEC) in CNS [23]. In the present study, Opm31 significantly promoted secretion of cytokines (IL-6, TNF-α, IL-1β and IL-10) in the supernatant of astrocytes compared with PBS control group.

Bcl-2 and Bax have the opposite effects and play vital roles in the mitochondrial pathway of apoptosis [26]. Cyt-C, distributed in the mitochondrial membrane, is an important aspect of the intrinsic pathway of apoptosis [27]. Zhao *et al*. reported that the levels of Cyt-C, caspase-9 and caspase-3 were closely related to pentavalent vanadium-induced neuronal apoptosis [28]. In this study, flow cytometry results showed that Opm31 significantly suppressed astrocytes apoptosis by the decrease of Bcl-2/Bax ratio. In addition, the release of Cyt-C from the mitochondrial membrane into the cytoplasm was also induced by Opm31. Besides, the apoptosis was further confirmed by the activation of caspase-3. All the results suggested that Opm31 significantly inhibited apoptosis of astrocytes.

p38MAPK, a serine/threonine protein kinase, is a crucial signaling pathway activated by extracellular stimulation. p38MAPK can regulate various cellular biological activities, such as inflammatory reactions, cell growth and apoptosis, and control cellular responses to cytokines and stress [29-31]. In the present study, Opm31 significantly improved the levels of p-38MAPK and p-MK2 and activated the p38MAPK signaling pathway. To further demonstrate the relationships between the p38MAPK signaling pathway and Opm31, p38MAPK genetic silencing by si-p38MAPK was used to inhibit p38MAPK expression, which significantly decreased Opm31-induced apoptosis rates. SB203580, a selective p38MAPK inhibitor, also significantly suppressed apoptosis rates triggered by Opm31 in astrocytes. All the results suggested that Opm31 induced cell apoptosis via the p38MAPK/MK2 signaling pathway in astrocytes.

Taken together, the present study suggested that *B. melitensis* Opm31 promoted the secretion of inflammatory cytokines and induced the apoptosis of astrocytes via p38MAPK/MK2 signal pathway, providing a potential pathogenesis of neurobrucellosis infected by *Brucella* in astrocytes.

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Disclosure of conflict of interest

None.

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