Supplementary information

Aeromonas sobria regulates proinflammatory immune response in mouse macrophages via activating the MAPK, AKT, and NF-κB pathways

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Materials and methods

1 Bacterial strains and mice

A. sobria strain ATCC 43979 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 30°C in nutrient broth. Six–eight-week-old specific-pathogen-free C57BL/6 female mice were purchased from Pizhou Oriental Breeding Company. The mice were housed in filter-top cages under a 12:12-h light/dark cycle and with *ad libitum* access to sterile drinking water and SPF-level mouse feed. All animal experiments were strictly performed according to the guidelines for the Animal Welfare and Research Ethics Committee of Jiangsu Ocean University (Permit Number: 2017124242).

2 Isolation and culture of primary mouse peritoneal macrophages (PMφ)

Mice were intraperitoneally injected with 2.5 mL of sterile Difco fluid thioglycollate medium (BD, New Jersey, USA). After 3–4 d, the mice were euthanized through cervical dislocation, and macrophages enriched in the peritoneal cavity were harvested. After centrifugation at 1000×*g* for 10 min, the PMφ were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Biological Industries, Beit-Haemek, Israel) and cultured in 6-well plates (Thermo Fisher, USA) at a density of 4.5×10^6 cells/well at 37 °C with 5% CO_2. After sedimentation for 6 h, nonadherent cells were removed, and incubation was continued for 12 h before stimulation.

3 Enzyme-linked immunosorbent assays (ELISAs)

The supernatants were collected from *A. sobria* infected PMφ at 12 h. The inflammatory cytokines secretion levels of IL-1β, TNF-α, IL-12, and IL-6 in the supernatants were determined using corresponding mouse ELISA kits (Invitrogen, USA). Detailed procedures were as previously described (Zhao et al., 2021).

4 Western blotting

A. sobria-infected PMφ pellets were collected at 0, 0.5, 1, 2, and 4 h, respectively. Then, the pellets were lysed using protease and phosphatase inhibitor contained RIPA buffer and quantified using BCA method. The prepared protein samples (20 μg per well) was separated by SDS-PAGE and transferred to 0.45 μm PVDF membrane (Millipore, USA). After blocking in 5% nonfat milk, the membranes were incubated overnight with primary antibody at 4 °C. Those primary antibodies are from MAPK signal pathway (p38, phospho-p38, ERK, phospho-ERK, JNK, phospho-JNK), AKT signal pathway (AKT, phospho-AKT), and NF-κB signal pathway (phospho-p65, IκBα, phospho-IKKα/β). All these antibodies were bought from Cell Signaling Technology (USA) and diluted 1,000 times in 5% nonfat milk when use. The internal protein β-actin was obtained from Proteintech (Wuhan, China) and diluted 5,000 times when use. After washing three times in 1× TBST washing buffer, the membranes were incubated with HRP-conjugated secondary detection antibodies (Cell Signaling Technology, USA) at a dilution of 1:1,000 under conditions of room temperature and 1 h. The immunoblots on the membranes were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and imaged with western blot imaging system (Clinx, Shanghai). The quantification analysis by measuring gray value was performed using ImageJ software.

5 Inhibition assays

To determine whether these activated signaling pathways involved in *A. sobria*-induced inflammatory response in PMφ, inhibition assays were carried out. In detail, PMφ were incubated with MAPK signaling pathway inhibitors (30 μM SB203580 for p38 target, 300 nM SCH772984 for ERK target, 10 μM SP600125 for JNK target, MedChemExpress, USA), AKT signaling pathway inhibitor (5 μM MK-2206 2HCl for AKT1/2/3 target, Selleck, Shanghai, China), and NF-κB signaling pathway inhibitor (5 μM BAY 11-7082 for IκBα target) for 2 h. After washing three times using sterile 1× TBS buffer, PMφ were stimulated with *A. sobria* for 12 h and the supernatants were collected for ELISA assays.

6 Immunofluorescence staining

The PMφ were cultured on sterile glass coverslips and stimulated with *A. sobria* at a multiplicity of infection (MOI) of 10 for 6 h. The cells were subsequently fixed in 4% paraformaldehyde (Biosharp, Beijing, China) at RT for 10 min, permeabilized in 0.1% Triton X-100 at RT for 20 min, blocked in 5% BSA at RT for 2 h, and incubated with rabbit anti-phospho-NF-κB p65 antibody (1:100) at 4°C overnight. The next day, the samples were incubated with FITC AffiniPure goat anti-rabbit IgG (H+L) antibody (1:100, Earthox, USA) at 37°C for 1 h followed by counterstaining with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1 μg/mL, Thermo Scientific, USA). NF-κB p65 subcellular localization was observed using a fluorescence microscope (Olympus, Japan).

7 Statistical analysis

Data are presented as means±SD from three independent assays with three technical repeats.

Differences between two groups were analyzed using a Student's *t*-test. Differences among three or more groups were evaluated using one-way analysis of variance (ANOVA). *P*-values of <0.05 were considered statistically significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

References

Zhao, P., Cao, L., Wang, X., *et al.*, 2021. *Giardia duodenalis* extracellular vesicles regulate the proinflammatory immune response in mouse macrophages *in vitro* via the MAPK, AKT and NF-κB pathways. *Parasite. Vector.,* 14(1), 358. doi:10.1186/s13071-021-04865-5