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

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Aeromonas sobria, a Gram-negative bacterium that can colonize both humans and animals, is found in a variety of environments, including water, seafood, meat, and vegetables (Cahill, 1990; Galindo et al., 2004; Song et al., 2019). *Aeromonas* spp. are conditionally pathogenic bacteria in aquaculture, which can rapidly proliferate, causing disease and even death in fish, especially when the environment is degraded (Neamat-Allah et al., 2020, 2021a, 2021b). In developing countries, *Aeromonas* spp. have been associated with a wide spectrum of infections in humans, including gastroenteritis, wound infections, septicemia, and lung infections (San Joaquin and Pickett, 1988; Wang et al., 2009; Su et al., 2013). Infections caused by *Aeromonas* spp. are usually more severe in immunocompromised individuals (Miyamoto et al., 2017). The presence of a plasmid encoding a β-lactamase in *A. sobria* that confers resistance to β-lactam antibiotics poses a huge challenge to the treatment of diseases caused by this microorganism (Lim and Hong, 2020). Consequently, an in-depth understanding of the interaction between *A. sobria* and its hosts is urgently required to enable the development of effective strategies for the treatment of *A. sobria* infections.

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Innate immunity represents the first line of defense against pathogen invasion through the recognition of a vast array of pathogen-associated or danger-associated molecular patterns released by invading microbes. Macrophages as the most important phagocytic cells are widely present in mammals and play key roles in the elimination of pathogens (Wang XC et al., 2019). *Aeromonas hydrophila* has been reported to trigger host proinflammatory responses through the activation of caspase-1 and the release of interleukin-1β (IL-1β) (McCoy et al., 2010). Other studies have shown that this bacterium can induce the activation of mitogen-activated protein kinase (MAPK) signaling and classical caspase-associated apoptosis in macrophages and intestinal epithelial cells (Galindo et al., 2004). *Aeromonas veronii* infection was shown to activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome in mouse macrophages, leading to apoptosis (McCoy et al., 2010). *A. sobria* bacteremia was associated with the highest mortality among members of the genus *Aeromonas* (56%) (Martins et al., 2002). However, the mechanisms underlying innate immune responses against *A. sobria* infection in mammals remain elusive. The MAPK signaling pathway includes a variety of kinases, namely, p38 MAPK, extracellular signal-related kinase (ERK), and c-Jun-activated kinase (JNK), that play significant roles in cell development and differentiation, as well as in controlling cell responses to cytokines and stress (Lewis et al., 1998; Garrington and Johnson, 1999; Morrison, 2012). Nuclear factor-κB (NF-κB), a member of the Rel family of nuclear

transcription factors, regulates the expression of a set of important genes in a wide variety of biological processes, such as innate immunity, adaptive immunity, inflammation, and stress responses (Perkins, 2006). The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway, which can be activated by insulin or a variety of growth and survival factors, has essential functions in controlling cell survival and apoptosis (Franke et al., 1995, 1997). Whether these signaling pathways are activated during *A. sobria* infection and involved in the interaction between *A. sobria* and macrophages needs to be further explored; such in-depth research on the mechanisms implicated in *A. sobria*-induced inflammation may provide new targets for the treatment of *A. sobria*-associated infections.

Therefore, in the present study, we established an *A. sobria* infection model using primary mouse peritoneal macrophages (PMφ); measured proinflammatory cytokine expression levels using quantitative real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) methods; researched the roles of p38, ERK, JNK, AKT, and NF-κB signaling pathways through inhibition assays; explored the subcellular localization of NF-κB p65 by immunofluorescence assay; and found that *A. sobria* induced inflammatory responses through the activation of the MAPK and NF-κB signaling pathways.

In order to evaluate the effects of *A. sobria* on PMφ, the levels of secreted proinflammatory cytokines in the cell supernatants were determined after 12 h of stimulation with *A. sobria* at a multiplicity of infection (MOI) of 10. As shown in Fig. 1, the secretion levels of IL-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF-α) were significantly upregulated (P<0.001) compared with those of the control (unstimulated) group. These data revealed that *A. sobria* triggered an inflammatory response in PMφ.

The MAPK signaling pathway is involved in immune responses triggered by a variety of pathogens. To explore the roles of this signaling pathway in *A. sobria*-induced proinflammatory cytokine production, mouse macrophages were inoculated with *A. sobria*, and cells were collected at 0, 0.5, 1.0, 2.0, and 4.0 h for the determination of total protein and phosphorylated protein (p38 MAPK, JNK, and MAPK (ERK1/2)) expression levels using western blotting (Fig. 2a). As shown in Fig. 2b, the expression of phospho-p38 MAPK (P-p38) was significantly upregulated compared with uninoculated controls at the time points of 0.5, 1.0, and 2.0 h (P<0.05), and was highly upregulated in PMφ 4.0 h post *A. sobria* infection (P<0.001). The levels of phospho-JNK (P-JNK) were significantly higher at all assessed time points, but the change was the most notable at 0.5 h (P<0.001; Fig. 2d). The gray analysis showed that phospho-ERK (P-ERK) protein expression was highly upregulated (P<0.001) at 0.5 and 1.0 h after *A. sobria* inoculation, but gradually decreased thereafter; however, the differences remained significant in comparison with the levels in uninfected cells (P<0.01) (Fig. 2c).

In order to determine the roles of the activated MAPK signaling pathway in *A. sobria*-mediated inflammatory responses in PMφ, inhibition assays were conducted with pathway-specific inhibitors (SB203580, SCH772984, and SP600125). Cells were incubated with inhibitors prior to *A. sobria* infection, and the supernatants

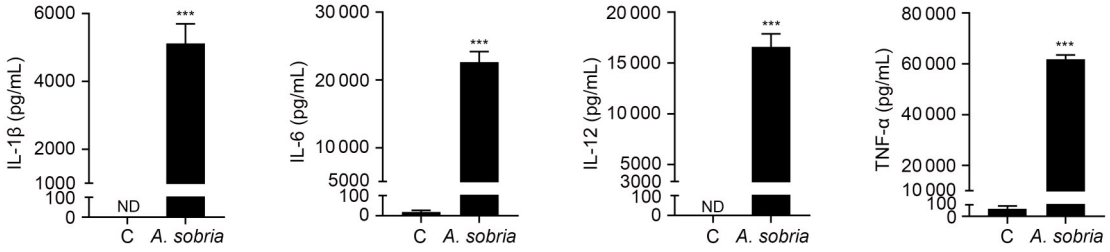

Fig. 1 Inflammatory responses of PMφ triggered by *Aeromonas sobria*. The PMφ (4.5×10^6 cells/well in a six-well plate) were inoculated with *A. sobria* (MOI=10) for 90 min. After washing three times with sterile PBS, the cells were treated with RPMI 1640 maintenance medium containing 100 μg/mL gentamicin sulfate for 2 h, and then with RPMI 1640 maintenance medium containing 20 μg/mL gentamicin sulfate for 12 h. The culture supernatants were subsequently collected for the determination of proinflammatory cytokine levels (IL-1β, IL-6, IL-12, and TNF-α). The recorded data are presented as mean±SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. *** P<0.001 was set as statistical significance in comparison with the control group (C). PMφ: peritoneal macrophages; MOI: multiplicity of infection; PBS: phosphate-buffered saline; RPMI: Roswell Park Memorial Institute; IL: interleukin; TNF: tumor necrosis factor; SD: standard deviation; ND: not detected.

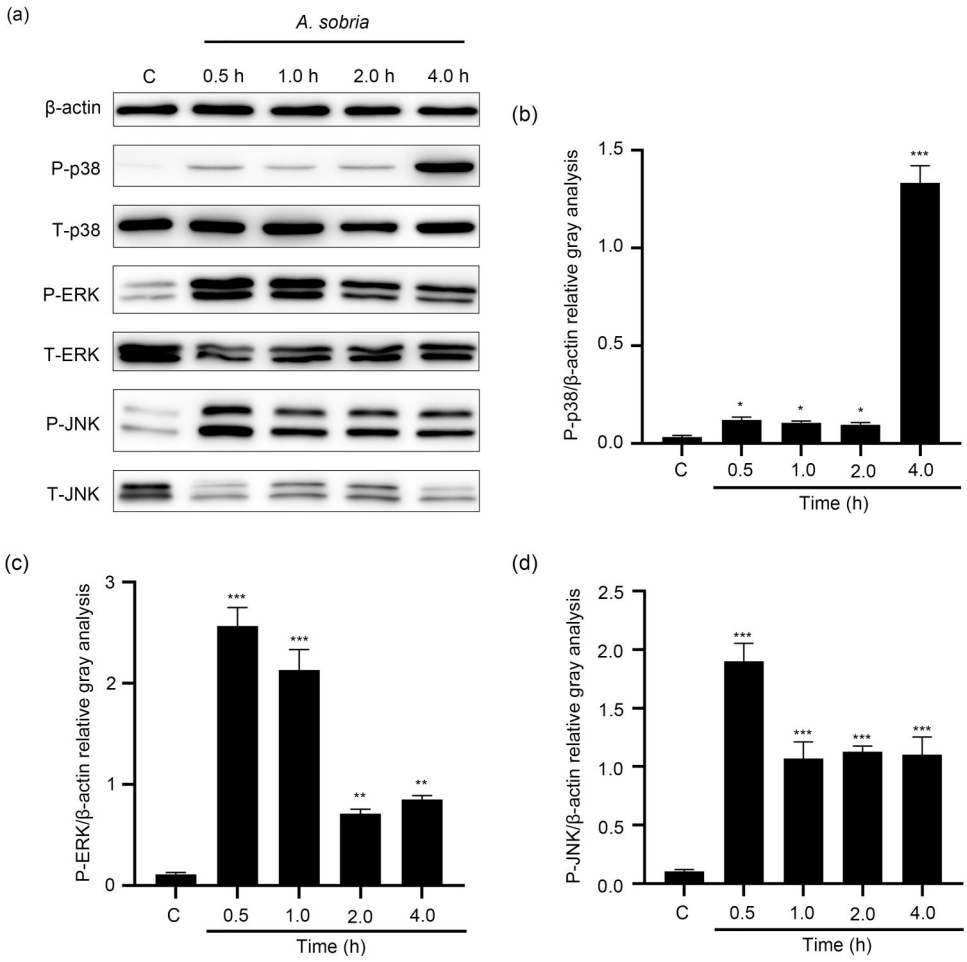

Fig. 2 Activated p38 and ERK signaling pathways in PMφ by *Aeromonas sobria* infection. (a) PMφ (4.5×10^6 cells/well in a six-well plate) were inoculated with *A. sobria* (MOI=10). The cells lysates were collected at 0.5, 1.0, 2.0, and 4.0 h for western blot analysis using primary antibodies against p38, P-p38, ERK, P-ERK, JNK, P-JNK, and β-actin. HRP-conjugated goat anti-rabbit IgG or horse anti-mouse IgG served as the secondary antibody. (b–d) Phosphorylation protein bands (P-p38 (b), P-ERK (c), and P-JNK (d)) were quantified using ImageJ software, and relative grayscale analysis was conducted through normalizing to β-actin. The data are presented as mean±SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, vs. the control group (C). MAPK: mitogen-activated protein kinase; PMφ: peritoneal macrophages; MOI: multiplicity of infection; P-: phospho-; T-: total-; ERK: extracellular signal-related kinase; JNK: c-Jun-activated kinase; HRP: horseradish peroxidase; IgG: immunoglobulin G; SD: standard deviation.

were collected for the determination of proinflammatory cytokine levels. As shown in Figs. 3a–3d, the treatment with either SB203580 or SP600125 led to a significant reduction in the levels of secreted IL-1β, IL-6, IL-12, and TNF-α ($P<0.05$, $P<0.01$, or $P<0.001$). Although SCH772984 treatment also decreased the levels of these cytokines, only IL-1β showed a significant reduction ($P<0.01$; Fig. 3a). These results revealed that the MAPK signaling pathway was activated in response to *A. sobria* infection, and the p38 MAPK and JNK pathways were involved in *A. sobria*-mediated inflammation in PMφ.

Next, we examined whether the PI3K/AKT signaling pathway, an important regulator of cell survival and apoptosis, was involved in *A. sobria*-mediated inflammation in PMφ. The total AKT protein and phospho-AKT (P-AKT) protein levels were quantified using ImageJ software. The results showed that P-AKT levels were significantly increased ($P<0.001$) at the time points of 0.5, 1.0, and 2.0 h, and then gradually decreased after 4.0 h ($P<0.05$; Figs. 4a and 4b).

In order to explore whether a correlation existed between the activated AKT pathway and *A. sobria*-mediated inflammatory response in PMφ, the

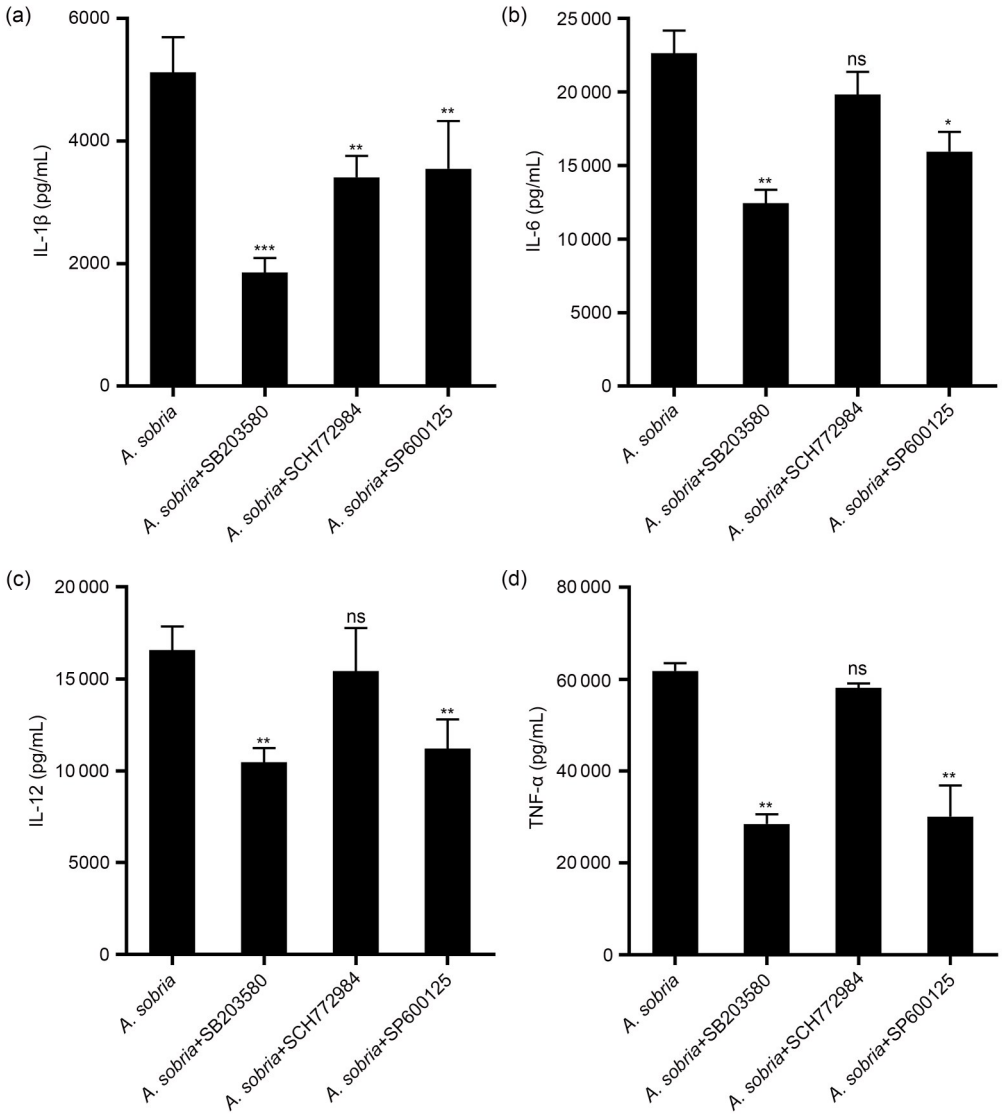

Fig. 3 *Aeromonas sobria* infection enhanced the PMφ inflammatory response through the phosphorylation of p38 and ERK. The cell culture supernatants were pretreated with the inhibitors SB203580, SCH772984, and SP600125 to final concentrations of 30 μmol/L, 300 nmol/L, and 10 μmol/L, respectively. Next, PMφ were inoculated with *A. sobria* for 12 h. Subsequently, the supernatants were collected for the determination of the levels of proinflammatory cytokines IL-1β (a), IL-6 (b), IL-12 (c), and TNF-α (d). The data are presented as mean±SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, ^{ns}P>0.05, vs. the control group (without pretreatment). ns: statistically insignificant; PMφ: peritoneal macrophages; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-related kinase; IL: interleukin; TNF: tumor necrosis factor; SD: standard deviation.

macrophages were pretreated with the inhibitor MK-2206 2HCl and then inoculated with *A. sobria*. The levels of all assessed proinflammatory cytokines (IL-1β, IL-6, IL-12, and TNF-α) were found to be significantly elevated to a varying extent (P<0.001, P<0.05, P<0.05, and P<0.01, respectively) compared with those of uninoculated controls (Figs. 5a–5d). These findings indicated that the AKT signaling pathway negatively regulated *A. sobria*-induced inflammation in PMφ.

Since the NF-κB signaling pathway is known to regulate the expression of numerous genes following cytokine stimulation, we explored its role in *A. sobria*-triggered inflammation in PMφ. The subcellular localization analysis of NF-κB p65 by immunofluorescence assay showed that P-p65 was localized in the cytoplasm in control PMφ cells; however, following *A. sobria* stimulation, P-p65 fluorescence could be clearly observed in the nucleus (Fig. 6a). The western blot analysis

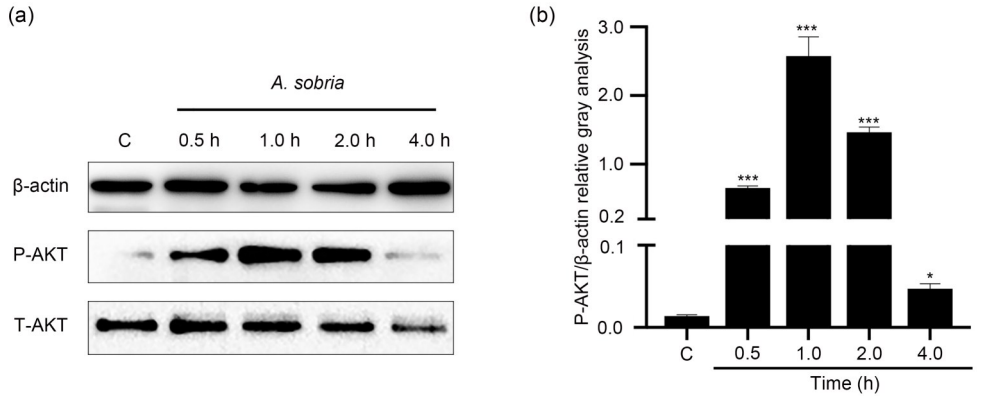

Fig. 4 ***Aeromonas sobria*** **infection activated the AKT signaling pathway in PMφ. (a) PMφ (4.5×10^6 cells/well in a six-well plate) were inoculated with** ***A. sobria*** **(MOI=10). The cells lysates were collected at 0.5, 1.0, 2.0, and 4.0 h for western blot analysis using primary antibodies against AKT, P-AKT, and β-actin. HRP-conjugated goat anti-rabbit IgG or horse anti-mouse IgG served as the secondary antibody. (b) The phosphorylation of AKT protein bands was quantified using ImageJ software, and relative grayscale analysis was made by normalizing to β-actin. The data are presented as mean± SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. $^*P$<0.05, $^{***}P$<0.001, vs. the control group (C). P-: phospho-; T-: total-; AKT: protein kinase B; PMφ: peritoneal macrophages; MOI: multiplicity of infection; HRP: horseradish peroxidase; IgG: immunoglobulin G; SD: standard deviation.**

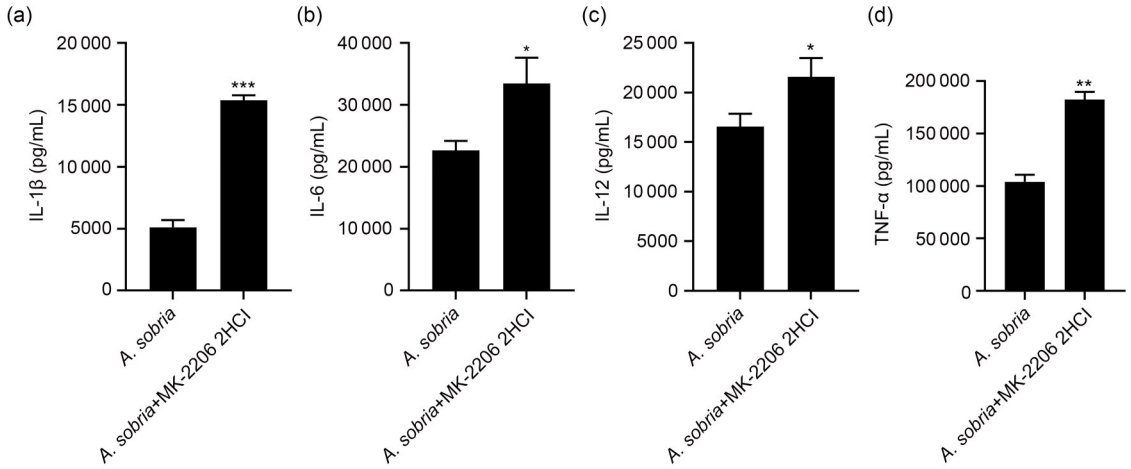

Fig. 5 ***Aeromonas sobria*** **infection decreased the PMφ inflammatory response through the phosphorylation of AKT. The cell culture supernatants were pretreated with the inhibitor MK-2206 2HCl to a final concentration of 5 μmol/L, and the PMφ were inoculated with** ***A. sobria*** **for 12 h. The supernatants were then collected for the determination of the levels of proinflammatory cytokines IL-1β (a), IL-6 (b), IL-12 (c), and TNF-α (d). The data are presented as mean±SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, vs. the control group (without pretreatment). PMφ: peritoneal macrophages; AKT: protein kinase B; IL: interleukin; TNF: tumor necrosis factor; SD: standard deviation.**

showed that the levels of P-p65 were significantly increased at the 0.5 h collection time point and peaked at 4.0 h compared with uninoculated control (P<0.001; Figs. 6b and 6c). In contrast, the protein levels of inhibitor of κBα (IκBα) significantly decreased with the infection time, reaching their minimum at 4.0 h (P< 0.001; Figs. 6b and 6d). Moreover, similarly to P-p65, the levels of phosphorylated inhibitor of κB kinase α/β (P-IKKα/β) increased with prolonged infection time (P<0.05 and P<0.001) and peaked at 4.0 h (Figs. 6b and 6e).

Then, the effects of blocking the NF-κB signaling pathway on proinflammatory cytokine levels in *A. sobria*-treated PMφ were investigated using the inhibitor BAY11-7082. Blocking the NF-κB pathway extremely significantly downregulated the expression of IL-1β, IL-6, and TNF-α (P<0.001; Figs. 7a, 7b, and 7d), and markedly reduced that of IL-12 (P<0.01; Fig. 7c).

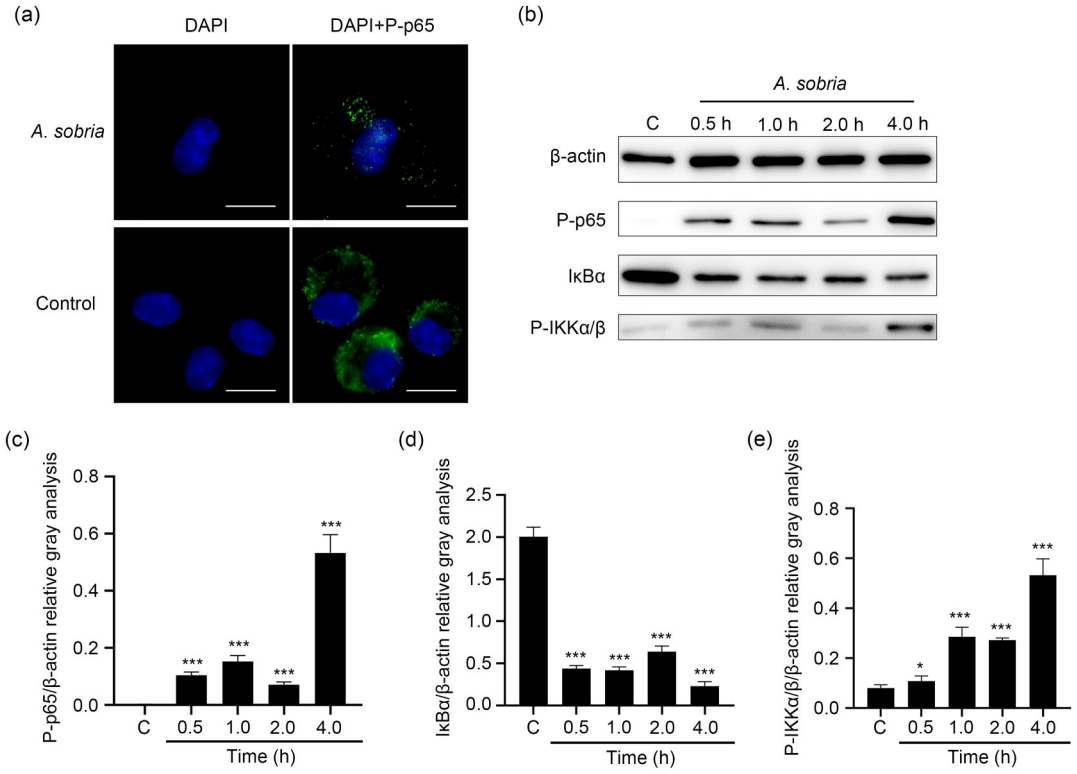

Fig. 6 *Aeromonas sobria* infection activated the NF-κB signaling pathway in PMφ. (a) PMφ (5×10^5 cells/well in a 24-well plate) were inoculated with *A. sobria* (MOI=10) and cultured for 4.0 h. The cells were then incubated with a primary antibody targeting P-NF-κB p65 followed by FITC-labeled goat anti-rabbit IgG secondary antibody. The nucleus was counterstained with DAPI (1 μg/mL). Uninfected cells were used as control. Scale bar=10 μm. (b) PMφ (4.5×10^6 cells/well in a six-well plate) were inoculated with *A. sobria* (MOI=10). The cells lysates were collected at 0.5, 1.0, 2.0, and 4.0 h for western blotting analysis using primary antibodies against P-NF-κB p65, IκBα, P-IKKα/β, and β-actin. HRP-conjugated goat anti-rabbit IgG or horse anti-mouse IgG served as the secondary antibody. Protein bands of P-NF-κB p65 (c), IκBα (d), and P-IKKα/β (e) were quantified using ImageJ software and relative grayscale analysis was made by normalizing to β-actin. The data are presented as mean±SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. $^{*}P<0.05$, $^{***}P<0.001$, vs. the control group (C). NF-κB: nuclear factor-κB; PMφ: peritoneal macrophages; MOI: multiplicity of infection; P-: phospho-; FITC: fluorescein isothiocyanate; IgG: immunoglobulin G; DAPI: dihydrochloride; IκBα: inhibitor of κBα; IKKα/β: inhibitor of κB kinase α/β; HRP: horseradish peroxidase; SD: standard deviation.

Overall, these results revealed that the NF-κB signaling pathway is actively involved in the *A. sobria* infection process in PMφ, and it enhances the secretion of proinflammatory cytokines.

Aeromonas spp. are becoming more widely recognized as human pathogens, particularly in developing countries and in immunocompromised individuals suffering from diseases such as cancer, liver cirrhosis, and diabetes (Parker and Shaw, 2011). Moreover, *Aeromonas* infection has been associated with a high fatality rate ranging from 25% to 70% in bacteremia-affected immunocompromised patients (Figueras et al., 2007). Among members of the genus *Aeromonas*, *A. sobria* was reported as the most virulent species in cases of bacteremia. Nevertheless, the mechanisms underlying the associated pathogenesis have not been clarified (Martins et al., 2002). In this study, we explored the effects of *A. sobria* on innate immunity using primary mouse PMφ and investigated the potential underlying mechanisms to provide a reference for future studies on the treatment of *A. sobria* infections in mammals.

Research has indicated that the aerolysin-related cytotoxic enterotoxin of *A. hydrophila* can activate the NF-κB signaling pathway in murine macrophages. Furthermore, proinflammatory genes controlled by the NF-κB pathway were upregulated in the gills of rainbow trout infected by *Aeromonas salmonicida*, indicating that NF-κB pathway-mediated inflammation responses are involved in host defenses against this

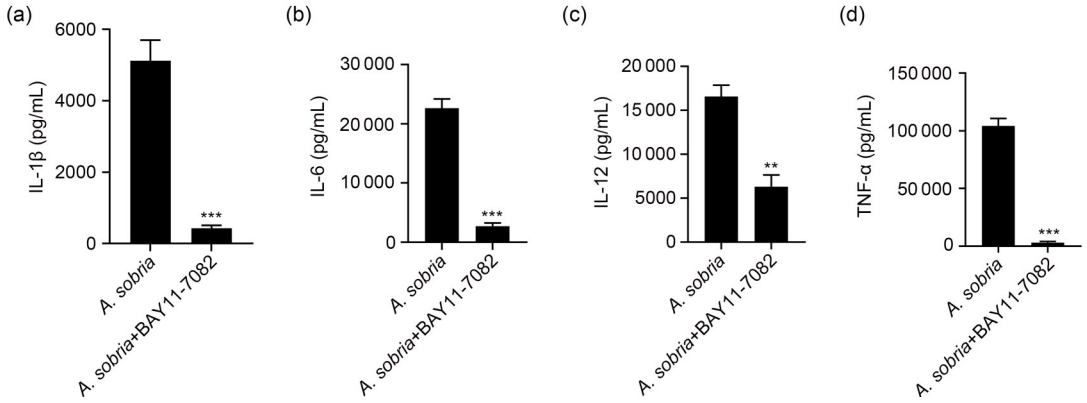

Fig. 7 *Aeromonas sobria* infection enhanced the PMφ inflammatory response through the activation of NF-κB signaling pathway. Cell culture supernatants were pretreated with the inhibitor BAY11-7082 to a final concentration of 5 μmol/L. Subsequently, PMφ were inoculated with *A. sobria* for 12.0 h, and the supernatants were collected for the determination of proinflammatory cytokine levels (IL-1β, IL-6, IL-12, and TNF-α). The data are presented as mean±SD of three independent assays, and the graphs were generated using GraphPad Prism 8 software. $^{**}P<0.01$, $^{***}P<0.001$, vs. the control group (without pretreatment). PMφ: peritoneal macrophages; NF-κB: nuclear factor-κB; IL: interleukin; TNF: tumor necrosis factor; SD: standard deviation.

microorganism (Wang WW et al., 2019). These observations demonstrate that proinflammatory cytokines are essential for organisms to eliminate and control *Aeromonas*-related infections in both humans and animals. In the current study, the levels of various proinflammatory cytokines were measured in PMφ infected with *A. sobria*. Similarly to those reported for other *Aeromonas* spp., the secretion levels of IL-1β, IL-6, IL-12, and TNF-α were significantly upregulated, which indicated that *A. sobria* triggered inflammatory responses in PMφ (Cahill, 1990; Galindo et al., 2004; Song et al., 2019).

The MAPK signaling cascades are crucial regulators of immune responses to infection by a variety of pathogens; they influence the levels of proinflammatory cytokines through the phosphorylation of p38, ERK, and JNK, and promote chromatin remodeling (Kirk et al., 2020). The cytotoxic enterotoxin of *A. hydrophila* has been shown to induce the phosphorylation of p38, JNK, and ERK kinases as well as cell apoptosis in both RAW 264.7 cells/murine PMφ and T84/HT-29 human intestinal epithelial cells (Galindo et al., 2004). The NF-κB signaling pathway is known to be involved in cellular stress responses, immunity, and inflammation (Perkins, 2006). The nuclear activity of NF-κB is controlled by IκB proteins, which provide transient or dynamic regulation through their stimuli-responsive degradation and resynthesis (Adelaja and Hoffmann, 2019). Moreover, studies have suggested that the transition from p50–p65 heterodimers to p50 homodimers in NF-κB may be related to inflammation (Lawrence et al., 2001; Ashall et al., 2009). We found that *A. sobria* activated the p38, ERK, and JNK pathways, while the inhibition assays indicated that only the p38 and JNK pathways played a promotive role in proinflammatory cytokine secretion. *A. sobria* infection decreased IκBα levels and promoted the phosphorylation of NF-κB p65, leading to its translocation into the nucleus and thus enhancing the proinflammatory response. In addition, *A. sobria* activated the AKT signaling pathway, and the inhibition of AKT phosphorylation increased proinflammatory cytokine levels. AKT is a serine/threonine-protein kinase with important roles in processes such as cell survival, proliferation, and metabolism (Lee et al., 2011). The opposing functions between the AKT and MAPK/NF-κB signaling pathways were consistent with the reported negative feedback roles of AKT in IL-12 production in dendritic cells (Fukao et al., 2002).

In summary, this study established an *A. sobria* infection model in vitro using primary mouse PMφ to explore the defense mechanisms involved in host cell response to *A. sobria* invasion. We found that *A. sobria* infection triggered proinflammatory cytokine production in PMφ through the activation of p38 MAPK, p44/42 MAPK (ERK1/2), and NF-κB signaling pathways, and this process was averted by the AKT signaling pathway. These findings may pave the way for the future exploration of immune response mechanisms associated with *A. sobria* infection in vivo.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Panpan ZHAO and Jingquan DONG conceptualized and designed the study. Wei ZHANG collected the data. Wei ZHANG, Bello Babatunde KAZEEM, Haitao YANG, Guanglu WANG, Zhixing LI, and Tao GUO performed the data processing and data analysis. Wei ZHANG, Bello Babatunde KAZEEM, and Panpan ZHAO wrote and edited the manuscript. Wei ZHANG and Bello Babatunde KAZEEM drew the figures. Jingquan DONG and Gang LIU checked the final version, and provided program finance support. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Wei ZHANG, Bello Babatunde KAZEEM, Haitao YANG, Gang LIU, Guanglu WANG, Zhixing LI, Tao GUO, Panpan ZHAO, and Jingquan DONG declare that they have no conflict of interest.

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). All institutional and national guidelines for the care and use of laboratory animals were followed.

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Supplementary information

Materials and methods