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# Aquaporin 9 promotes LPS-induced Eustachian tube inflammation through PP2A/NF-κB pathway

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ABSTRACT

Eustachian tube dysfunction (ETD) induced by Eustachian tube (ET) inflammation is an important cause to middle ear effusion and hearing impairment. Here, we found that aquaporin 9 (AQP9) was an important regulator of ET inflammation. AQP9 was upregulated during ET inflammation, while AQP9 deficiency alleviated LPS-induced ET inflammation by upregulating protein phosphatase 2A (PP2A) expression to suppress nuclear factor kappa-B (NF-κB) activation through P65 phosphorylation. In addition, pharmacological inhibition of AQP9 by phloretin similarly alleviated ET inflammation. These findings provide a new target for the treatment of ET inflammation to improve ETD.

## 1. Introduction

The Eustachian tube (ET), as a physiological channel connecting the middle ear and nasopharynx, plays crucial roles in pressure regulation, pathogen defense, and middle ear clearance [1]. Eustachian tube dysfunction (ETD) refers to the inability of the ET to open or close normally, resulting in an imbalance between the middle ear and the external pressure, causing a series of ear symptoms as self-hearing enhancement, ear fullness, and hearing loss, which seriously affects the quality of life [2]. ETD affects up to 40 % of children [3] and 5 % adults [4], is a major contributor for persistent Otitis media [5]. Pathogenic stimulation can trigger mucosal edema and hypersecretion, potentially leading to ET dysfunction [6]. This may result in middle ear effusion, hearing loss, and other complications that severely affect patients' quality of life [1,2]. Despite its clinical significance, the molecular mechanisms of ET inflammation remain poorly understood, and current treatments are limited. Thus, elucidating these pathogenic processes could provide insights into middle ear effusion pathogenesis and reveal the new therapeutic targets for ET inflammation and otitis media effusion (OME).

There are a variety of channel proteins with important functions in ET, including Aquaporin 9 (AQP9). AQP9 is an important membrane channel protein that mediates transmembrane movement of water, glycerol, hydrogen peroxide ($H_2O_2$), urea, ammonia, lactic acid, arsenite, and selenite. The multifunctional transport capability enables AQP9 to participate in diverse pathological processes including inflammation, tumorigenesis, and immune regulation [7,8]. Studies have found that AQP9 expresses in leukocytes modulating systemic inflammatory through regulating cell migration and phagocytosis. Its expression is upregulated in leukocytes, dendritic cells, neutrophils, and macrophages following lipopolysaccharide (LPS) stimulation [9], while *Aqp9* knockout significantly attenuates inflammatory responses [10], indicating its important role in inflammation progression. Phloretin is a non-specific inhibitor of aquaporins, and studies have found that phloretin can inhibit the expression of AQP9 [11]. Although early studies have detected AQP9 expression in ET [12], the role of AQP9 in ET inflammation has not been elucidated.

To our interest, AQP9 can promote the transmembrane movement of $H_2O_2$ [8], which has a regulatory effect on key proteins in inflammatory signaling pathways, such as protein phosphatase 2A (PP2A) [13]. PP2A is a ubiquitously expressed serine/threonine phosphatase with multiple important roles in cell signaling pathways. It participates in the inflammatory process of sepsis, chronic obstructive pulmonary disease and other diseases by regulating the phosphorylation of key proteins in inflammatory pathways such as nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) [14]. During inflammation,

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various signaling molecules can inhibit PP2A, leading to enhanced nuclear localization and phosphorylation of NF-κB, activation of p38 MAPK, and increased phosphorylation of tristetraprolin (TTP). These changes collectively promote the expression of multiple inflammatory mediators and contribute to tissue damage [15,16]. In contrast, reactivation of PP2A helps to suppress the progression of inflammation. Therefore, the AQP9, which has a role in mediating the transmembrane movement of $H_2O_2$, may have an important role in PP2A-related inflammatory signaling and warrants further investigation.

This study investigated the role of AQP9 in LPS-induced ET inflammation using *Aqp9* knockout mice. LPS stimulation upregulated AQP9 expression in ET. AQP9 promoted the accumulation of $H_2O_2$ in ET, inhibited PP2A, relieved the inhibition of PP2A on NF-κB phosphorylation and promoted its nuclear localization, and then activated various inflammatory factors expression. Whereas *Aqp9* knockout attenuated inflammation through PP2A/NF-κB pathway. Furthermore, pharmacological inhibition of AQP9 with Phloretin similarly suppressed LPS-induced ET inflammation, demonstrating therapeutic potential for ET-related inflammatory disorders and otitis media effusion (OME).

## 2. Methods

### 2.1. Animals

This study was conducted using C57BL/6 J mice (8 weeks old, about 20 g, male mice). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center (Approval No. DLASBD0161). Animal care complied with Chinese National Laboratory Animal Welfare Guidelines. *Aqp9*$^{-/-}$ mice (C57BL/6 background) generated via CRISPR/Cas9 technology were obtained from Peking University [17,18]. Wild-type (WT) C57BL/6 mice were acquired from the Peking University Health Science Center's Laboratory Animal Science Department. All mice were maintained under controlled environmental conditions (12-h light/dark cycle) with free access to food and water.

### 2.2. Intratympanic injection (IT)

To establish the ET inflammation model, mice were divided into two groups (6 in each group), and intratympanic injection (IT) [19,20] of LPS (Sigma, #L2630, Shanghai, China, IT, 2.5 mg/kg , 5 mg/mL) or 10 μL saline was performed for 48 h (Supplementary Fig. 1A).

To clarify the role of PP2A in AQP9-regulated ET inflammation, Mice were divided into two groups respectively (6 mice in each group), received injections of either PP2A inhibitor Okadaic acid (OA) (Cayman, #10011490, Beijing, China. IT, 10 μM) or 10 μL equivalent vehicle solution of Ethanol for 30 min (Supplementary Fig. 1B and Supplementary Fig. 4).

Exogenous $H_2O_2$ supplementation was used to explore the mechanism of AQP9 in ET inflammation. WT and *Aqp9*$^{-/-}$ mice were divided into two groups respectively (6 mice in each group) after the establishment of ET inflammation model, received injections of either PP2A inhibitor Okadaic acid (OA) (IT, 10 μM) or equivalent vehicle solution (10 μL) of Ethanol $H_2O_2$ (30 μM) and 10 μL control reagent were injected into the tympanic chamber for 30 min (Supplementary Fig. 1C).

To assess phloretin activity in anti-inflammation through AQP9 in ET, C57BL/6 mice were randomly grouped to three groups (6 in each group). The Control group receives methanol and intratympanic saline; LPS model group receives methanol and intratympanic LPS; Phloretin treatment group receives phloretin and intratympanic LPS. Either phloretin (Rhawn, #R013640, Beijing, China, 10 mg/kg) or equivalent vehicle solution (20 μL) of Methanol was received every Monday and Thursday intraperitoneal (i.p.) injections for 6 weeks (Supplementary Fig. 1D).

### 2.3. Hematoxylin & Eosin (H&E) staining

To observe the morphology of ET, ET was stored in 4 % PFA overnight, then was embedded in paraffin after decalcification for a month. And the paraffin sections (5 μm) were dewaxed and rehydrated in Gradient alcohol for the following H&E staining. H&E staining procedure was performed according to the manufacturer's instructions (Solarbio, #G1120, Beijing, China)

### 2.4. Western blotting (WB)

To detect the protein expression level, ET tissues were stored at −80 °C after harvesting. The ET was lysed in RIPA lysis buffer (Applygen, #C1053-100, Beijing, China) with Protease inhibitor (Applygen, #P1265, Beijing, China) and Phosphatase inhibitors (Cwbio, #CW2383S, Jiangsu, China). The supernatant was collected after protein centrifugation. The protein concentration was determined by the BCA kit (Applygen, #P1511, Beijing, China), and then the protein denaturation was performed with the protein loading buffer (Applygen, #P1040, Beijing, China). Equal quantities of protein were transferred to Nitrocellulose (NC) membrane after separated by 10 % SDS-PAGE electrophoresis, and then the NC membrane was sealed with 5 % skim milk and incubated with primary antibody at 4 °C overnight. AQP9 (1:1000, #sc-74409, Santa Cruz Biotechnology, USA), IL-1β (1:1000, #CSB-PA003023, Cusabio, China), COX2 (1:1000, CST, USA), P65 (1:1000, #6956T, CST, USA), p-P65 (1:1000, #YP0191, CST, USA), β-actin (1:1000, #3700, CST, USA). Secondary antibodies: Goat Anti-Rabbit IgG (H&L)-HRP Conjugated (1: 2000, # BE0101-100, Easybio, China), and Goat Anti-Mouse IgG (H&L)-HRP Conjugated (1:2000, # BE0102-100, Easybio, China) were used in this study. Protein expression was detected by enhanced chemiluminescence (Biodragon, #BF06053-50, Beijing, China) according to the manufacturer's suggests.

### 2.5. Real-time quantitative PCR (RT-qPCR)

To detect the gene transcription level, a FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, #RC101, Nanjing, China) was used for total RNA extraction from ET tissue. After measuring the RNA concentration by Nanodrop (Thermo, American), Extracted RNA (1000 ng) from each sample was used for complementary DNA (cDNA) synthesis with Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen, #11141ES60, Shanghai, China) following the manufacturer's instructions. cDNAs were amplified and quantified by Taq Pro Universal SYBR qPCR Master Mix (Vazyme, #Q712-02, Nanjing, China) on a Qs1 system following the manufacturer's advices. Data were normalized to the internal reference gene GAPDH and calculated as $2^{-[(\delta Ct \text{ of gene}) - (\delta Ct \text{ of GAPDH})]}$. The primer sequences were listed in the Supplementary Table 1.

### 2.6. Immunohistochemistry (IHC) staining

To detect the location and level of protein expression in ET, the 5 μm paraffin sections were hydrated with gradient ethanol, repaired with citric acid at 98 °C for 20 min, naturally restored to room temperature. Then the sections were washed with PBS containing 5 % goat serum and 0.5 % Triton X-100 after de-enzyme with 3 % $H_2O_2$. Next, the sections were incubated with primary antibody overnight. After the second antibody was incubated using Universal two-step Test Kit (Mouse/Rabbit Enhanced Polymer test system) (ZSGB-BIO, #PV900, China), the DAB kit (ZSGB-BIO, #ZLI-9081, China) was applied according to the manufacturer's suggestion. Gradient alcohol dehydration and neutral resin sealing were performed after hematoxylin re-staining. The images were observation under microscope. AQP9 (1:50, #sc-74409, Santa Cruz Biotechnology, USA), P65 (1:200, #6956T, CST, USA), p-P65 (1:200, #YP0191, CST, USA).

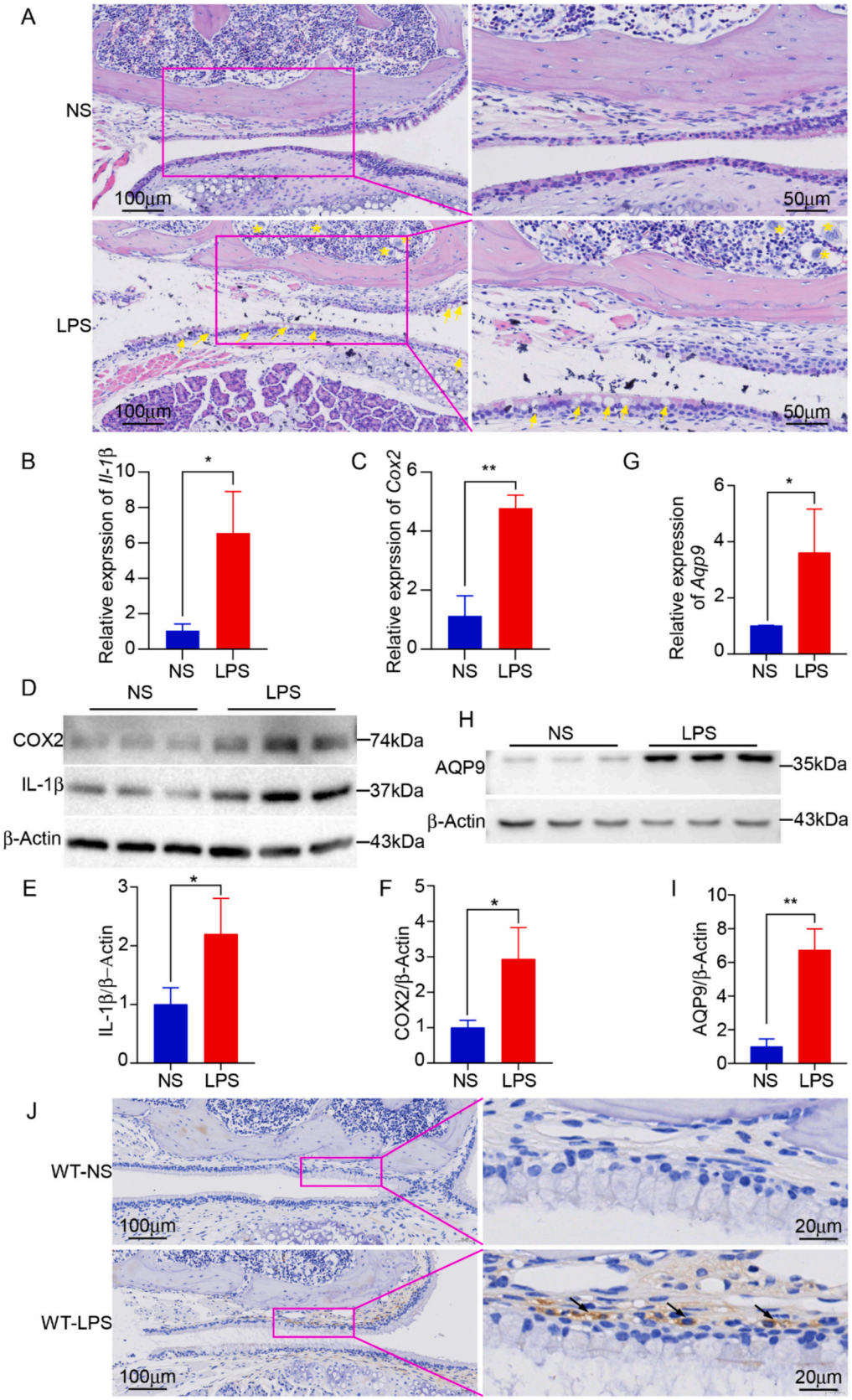


**Fig. 1.** The expression of AQP9 increased in LPS-induced ET inflammation. A, ET morphology. H&E staining showing mucosal hyperplasia, inflammatory infiltration (asterisks), and increased goblet cells (arrows) in LPS-treated ET. B-F, Inflammatory factor levels. qPCR and WB showed that IL-1β, and COX2 were increased in the LPS group. G-J, AQP9 levels. qPCR, WB and IHC showed that AQP9 was elevated in the LPS group.

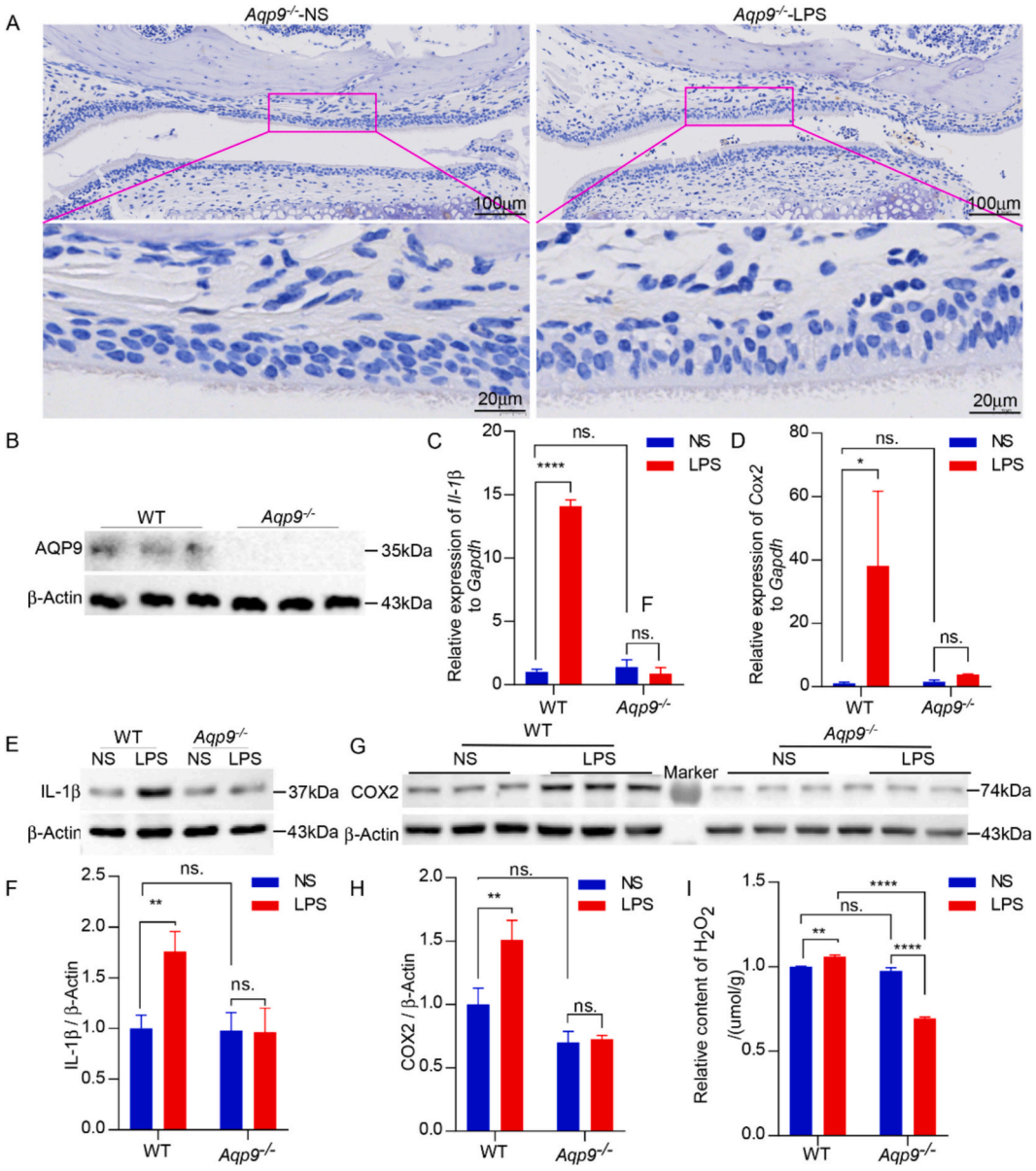


**Fig. 2.** *Aqp9*$^{-/-}$ attenuated LPS-induced ET inflammation and $H_2O_2$ accumulation. A. AQP9 expression in ET. IHC did not detect AQP9 in *Aqp9*$^{-/-}$ mice. B. AQP9 levels. WB did not detect AQP9 band in *Aqp9*$^{-/-}$ mice. C-H. Inflammatory factor levels. qPCR and WB showed that *Aqp9* knockout reversed LPS-induced up-regulation of IL-1β and COX2. I. $H_2O_2$ content detection. *Aqp9*$^{-/-}$ reversed the $H_2O_2$ accumulation in the ET induced by LPS.

### 2.7. Detection of hydrogen peroxide($H_2O_2$) content

To detect the content of $H_2O_2$, ET tissue was weighed and placed in acetone solution and homogenized on ice. After centrifugation (8000*g* for 10 min at 4 °C), the supernatant was collected on ice. The assay tube, standard tube and blank tube solutions were configured according to the instructions of the hydrogen peroxide ($H_2O_2$) content detection kit (Solarbio, #BC3595, Beijing, China). After 5 min at room temperature, 200 μL of each solution was taken into a 96-well plate to measure the absorbance value at 415 nm. The $H_2O_2$ content was calculated based on ET tissue mass.

### 2.8. Data analysis

Statistical analysis was performed by GraphPad Prism 8 software (GraphPad Software, USA). Each experiment was repeated for three times. Data was presented as the mean ± standard deviation (SD) and were analyzed with the two-tailed Student *t*-test or one/two-way analysis of variance (ANOVA), followed by multiple-comparison correction. *P* value of $<0.05$ was represented statistically significant.

## 3. Results

### 3.1. The expression of AQP9 increased in ET inflammation induced by LPS

We first performed intratympanic injection of LPS to induce ET inflammation. Histological analysis revealed ET mucosal swelling with inflammatory cell infiltration, heightened secretory cell activity, and mucus gland proliferation at 48 h post-injection (Fig. 1A). Concurrent upregulation of IL-1β and COX2 confirmed successful inflammation modeling (Fig. 1B-F). IL-1β and COX2 protein levels increased by 193 % and 124 %, respectively.

To clarify the expression of AQP9 in LPS-induced ET inflammation, RT-qPCR, western blot and immunohistochemical staining was conducted. Comparative analysis demonstrated significant AQP9

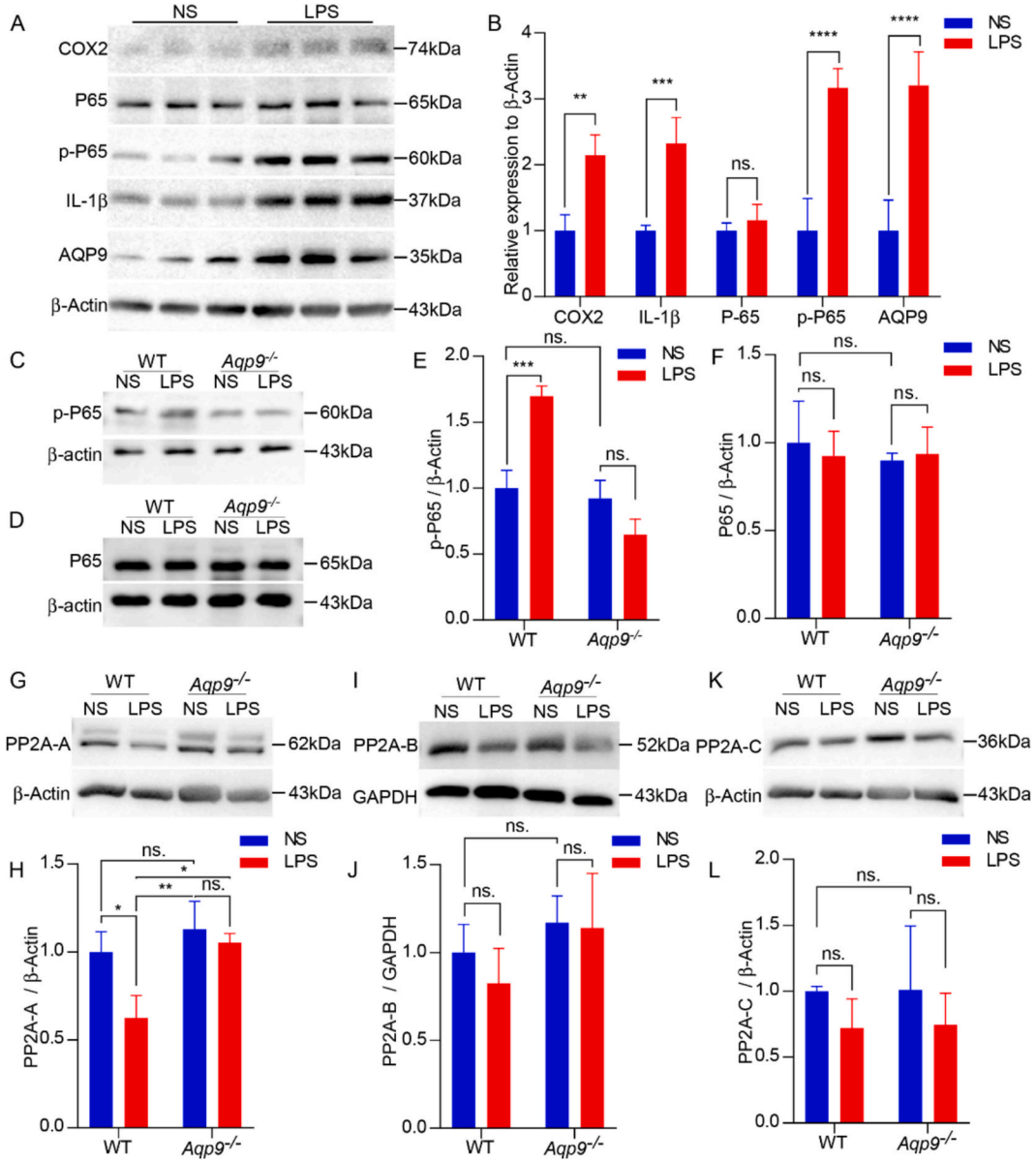


**Fig. 3.** *Aqp9* knockout inhibited LPS-induced activation of NK-κB signaling pathway and reversed down-regulation of PP2A-A. A-B, NF-κB signaling pathway detection in WT mice. Phospho-P65 and COX2/IL-1β were up-regulated in the LPS group. C-F, NF-κB detection in both WT and *Aqp9*$^{-/-}$ mice. LPS-induced phospho-P65 elevation that was reversed in *Aqp9*$^{-/-}$ mice. No changes were detected in total P65 levels. G-L, PP2A protein levels. LPS decreased PP2A-A in WT mice. PP2A-A level was up-regulated after *Aqp9* knockout. No changes were observed in PP2A-B/C subunits.

upregulation in LPS group (Fig. 1G-I), AQP9 was increased by 574 %. Immunohistochemical staining showed that AQP9 was obviously expressed in the LPS group and mainly located in submucosa of ET, closing to the tympanic cavity (Fig. 1J).

### 3.2. *Aqp9*$^{-/-}$ reversed LPS-induced ET inflammation and $H_2O_2$ accumulation

To investigate the role of AQP9 in ET inflammation, we used *Aqp9*$^{-/-}$ mice, which were successfully constructed early by our group [17,18], to observe its effect on ET inflammation. As is demonstrated, *Aqp9* was successfully knocked out (Fig. 2B). Immunohistochemistry staining showed that LPS did not cause AQP9 expression in ET of *Aqp9*$^{-/-}$ mice (Fig. 2A).

Comparison of inflammatory factors in ET between WT mice and *Aqp9*$^{-/-}$mice following LPS stimulation revealed that AQP9 deficiency reversed the LPS-induced upregulation of COX2 and IL-1β (Fig. 2C-H). This suggests that *Aqp9* knockout has an anti-inflammatory effect in ET infection. Additionally, LPS-induced $H_2O_2$ accumulation in ET was abolished in *Aqp9*$^{-/-}$ mice (Fig. 2I).

### 3.3. *Aqp9*$^{-/-}$ reversed LPS-induced ET inflammation by upregulating PP2A/NF-κB pathway

To clarify the mechanism of AQP9 in promoting ET inflammation induced by LPS, we analyzed phosphorylation of P65, a classical NF-κB pathway signal molecule. Phosphorylated P65 elevated significantly in LPS group (Fig. 3A-B), indicating that NF-κB pathway was activated. WT and *Aqp9*$^{-/-}$ mice then received intratympanic saline (IT-NS) or LPS (IT-LPS) to determine the anti-inflammatory mechanism of AQP9 deficiency. We found that AQP9 deficiency inhibited P65 phosphorylation (Fig. 3C-F). These results suggested that AQP9 might promote LPS-induced ET inflammation by activating NF-κB pathway.

In physiological conditions, inhibitor of NF-κB alpha (IκB-α) covered

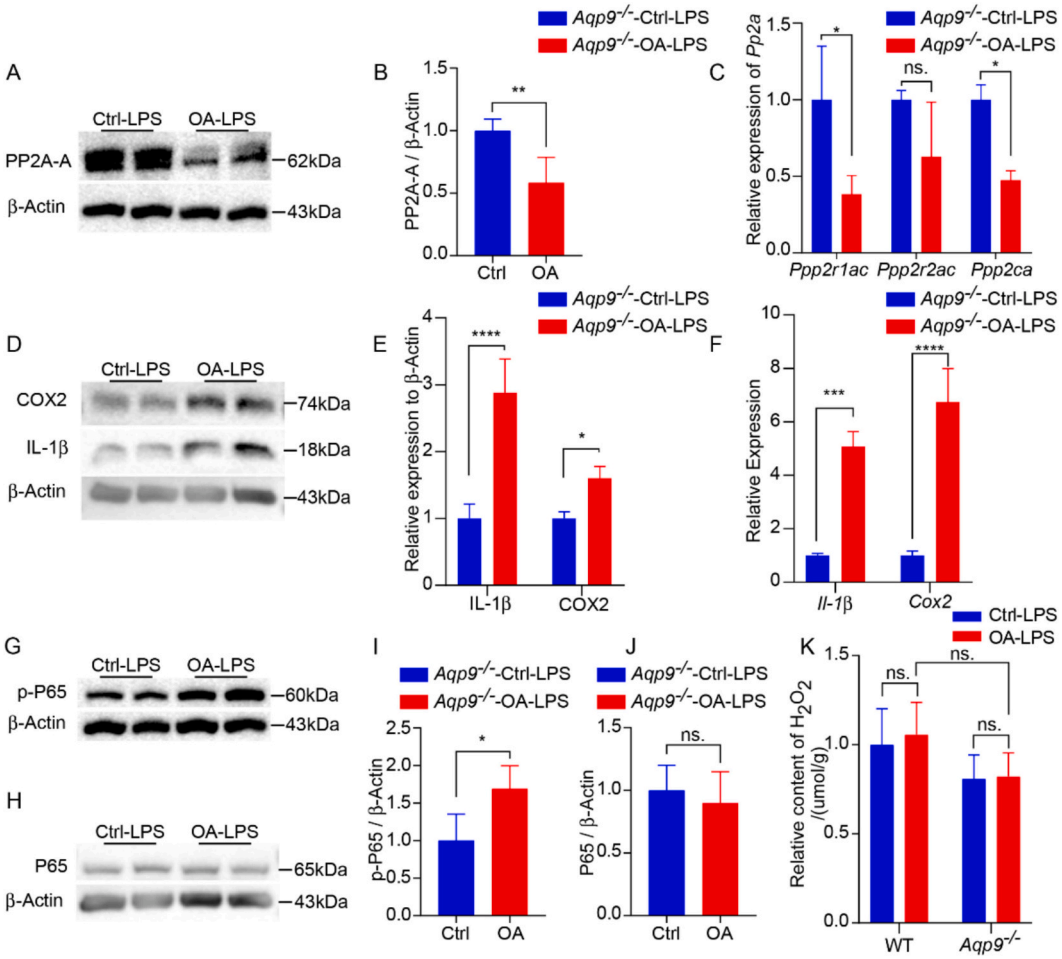


**Fig. 4.** OA inhibited PP2A and promoted P65 phosphorylation. A-B, PP2A-A modulation. PP2A-A was suppressed in OA-LPS intratympanic injection group in *Aqp9*$^{-/-}$ mice. C. OA effects. OA reduced *Ppp2r1ac/Ppp2r2ac/Ppp2ca* mRNA levels. D-F. Inflammatory factor levels after OA injection. OA treatment increased IL-1β/COX2 expression in *Aqp9*$^{-/-}$ mice. G-J. NF-κB pathway analysis. OA increased phospho-P65 levels in *Aqp9*$^{-/-}$ mice. No changes were detected in total P65 levels. K. $H_2O_2$ content detection. OA did not change $H_2O_2$ concentration both in WT and *Aqp9*$^{-/-}$ mice.

nuclear localization site of P65. When stimulated by LPS, IκB-α was phosphorylated, which promoted the degradation by proteasomal, and enabling P65 nuclear translocation [21,22]. PP2A could bind to IκB-α to inhibit its phosphorylation, thereby suppressing NF-κB activation [16]. To further clarify the function of AQP9 in ET inflammation, PP2A was examined in WT and *Aqp9*$^{-/-}$ mice. It was found that PP2A regulatory subunits (PP2A-A) were downregulated after LPS stimulation in WT mice. While after *Aqp9* knockout the expression of PP2A-A were upregulated (Fig. 3G-H), suggesting that increased AQP9 expression had an inhibitory effect on PP2A-A. There were no significant changes on PP2A structural subunits (PP2A-B) and catalytic subunits (PP2A-C) both in WT and Aqp9−/− mice (Fig. 3I-L). These results suggest that AQP9 may promote the activation of NF-κB signaling pathway by regulating PP2A-A.

### 3.4. PP2A inhibition reversed NF-κB activation and exogenous $H_2O_2$ failed to reverse LPS-induced ET inflammation in Aqp9-deficient mice

To further investigate the regulatory role of AQP9 through PP2A, we used PP2A inhibitor Okadaic acid (OA) to further investigate the regulatory role of AQP9. OA significantly suppressed the expression of PP2A-A subunit in *Aqp9*$^{-/-}$ mice (Fig. 4A-C, Supplementary Fig. 2) and reversed COX2, IL-1β (Fig. 4D-F) and phospho-P65 levels (Fig. 4G-J), supporting a mechanistic link between AQP9 and PP2A in controlling ET inflammation. However, OA did not affect $H_2O_2$ levels of ET in WT or *Aqp9*$^{-/-}$ mice (Fig. 4K). This indicates that PP2A does not exert upstream regulatory control over $H_2O_2$ levels. Instead, AQP9 appears to regulate intracellular $H_2O_2$ levels (Fig. 2I), which in turn influence PP2A activity and downstream inflammation.

As mentioned above, AQP9 deficiency reduced $H_2O_2$ accumulation in ET inflammation. Therefore, we further explored the role of AQP9 in regulating $H_2O_2$ transmembrane movement by exogenous supplementation of $H_2O_2$. To assess the role of $H_2O_2$ in AQP9-dependent inflammation, exogenous $H_2O_2$ (30 μM, 10 μL) or saline was administered to LPS-pretreated mice. Exogenous $H_2O_2$ failed to elevate COX2, IL-1β (Fig. 5A-C) and phospho-P65 levels (Fig. 5D-G) in both WT and *Aqp9*$^{-/-}$ mice, possibly because LPS had already induced maximal intracellular $H_2O_2$ accumulation, and AQP9 deficiency abolishes AQP9-mediated $H_2O_2$ transmembrane transport, leaving diffusion as the predominant $H_2O_2$ transport mechanism.

### 3.5. Phloretin ameliorated LPS-induced ET inflammation via AQP9 suppression

Phloretin has been shown the function of inhibiting aquaporins [11]. We found that phloretin significantly attenuated LPS-induced AQP9 expression (Fig. 6A, E-F), concurrently reducing $H_2O_2$ accumulation (Fig. 6P) and suppressing NF-κB activation (Fig. 6L-O). Phloretin upregulated PP2A-A transcription (Fig. 6B, G-H), and downregulated IL-1β and COX2 expression (Fig. 6C-D, I-K). These findings suggested that phloretin can alleviate ET inflammatory through AQP9 related PP2A/NF-κB signaling pathways.

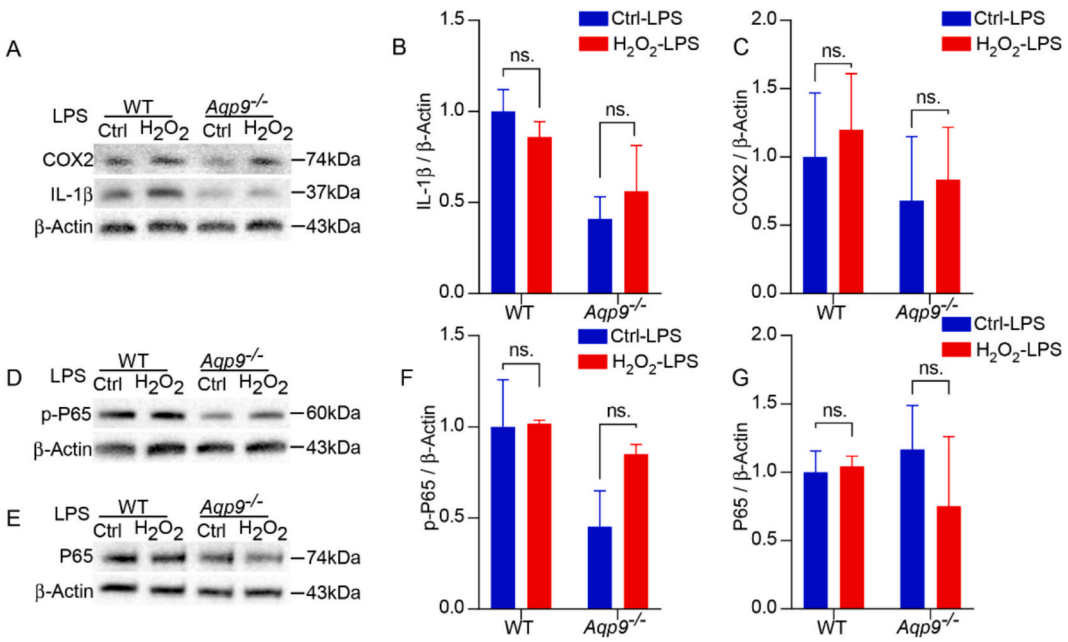


**Fig. 5.** Exogenous $H_2O_2$ fails to reverse LPS-induced inflammation in *Aqp9*$^{-/-}$ mice. A-C, Effect of exogenous $H_2O_2$ on inflammatory factors. Exogenous $H_2O_2$ did not change COX2/IL-1β levels both in *Aqp9*$^{-/-}$ mice. D-G, Effect of exogenous $H_2O_2$ on NF-κB activation. Exogenous $H_2O_2$ did not increase phospho-P65 levels after LPS-stimulation both in *Aqp9*$^{-/-}$ mice.

Based on the results, we believed that AQP9 promotes LPS-induced ET inflammation through PP2A/NF-κB pathway (Fig. 7). AQP9 promotes $H_2O_2$ accumulation in ET during LPS stimulation. The accumulation of $H_2O_2$ relieves the inhibitory effect of PP2A on NF-κB signaling pathway and promotes the phosphorylation of P65 into the nucleus. Then, the expression of inflammatory factors IL-1β and COX2 is upregulated, promoting ET inflammation. However, after *Aqp9* knockout, $H_2O_2$ could not be accumulated in ET, resulting in a sustained high level of PP2A, which inhibits the phosphorylation of P65 into the nucleus. It reduces the activation of NF-κB signaling pathway induced by LPS, and reduces ET inflammation finally. Administration of the PP2A inhibitor OA reversed the inhibitory effect of *Aqp9* knockdown. Phloretin could also ameliorate LPS-induced ET inflammation by inhibiting AQP9.

## 4. Discussion

ET is the unique physiological channel between middle ear and nasopharynx, the maintenance of ET function is critical. A variety of inflammatory factors such as respiratory infections, allergic rhinitis and chronic rhinitis [23–25] can cause ETD, but the mechanism of ETD is still unclear. Here, we identify AQP9 as an important regulator of LPS-induced ET inflammation, where its upregulation inhibits PP2A/NF-κB signaling to drive IL-1β/COX2 production and amplify inflammatory responses.

We found that AQP9 up-regulated on LPS-induced ET inflammation. AQP9 is a transmembrane aquaglyceroporin channel, and is upregulated in various inflammatory diseases. In patients with systemic inflammatory response syndrome (SIRS), AQP9 expression is increased in polymorphonuclear leukocytes (PMNLs), which induces morphological changes of leukocytes and chemotactic movement to inflammatory sites [26]. We found that AQP9 was distributed in ET submucosal macrophages (Supplementary Fig. 3) and was elevated in ET inflammation model induced by LPS (Fig. 1J). Researchers have also reported that AQP9 can promote macrophage chemotactic movement by increasing macrophage water flux, regulating local hydrostatic pressure and macrophage polarization [27,28]. In addition, researchers have also found that AQP9 can regulate metabolism or oxidative stress through $H_2O_2$ or glycerol transport, affecting inflammatory process or tissue regeneration [18,29,30]. *Aqp9* knockout has also been reported to play an important anti-inflammatory role. Previous studies have shown that AQP9 deficiency improves survival in endotoxin shock models [10] and attenuates NLRP3-mediated intestinal inflammation via suppression of the p38 MAPK pathway [31], which is consistent with our finding that *Aqp9* knockout inhibits inflammation in ET. Post-translational modifications of proteins, such as phosphorylation, glycosylation and ubiquitination, affect their localization and function. We found that AQP9 had a molecular weight of about 35 kDa in WT mice and was not found in *Aqp9* knockout mice (Fig. 2B), suggesting that there might be multiple post-translational modifications of AQP9 in ET.

We also found that AQP9 promoted $H_2O_2$ accumulation and ET inflammation. $H_2O_2$ is an important intracellular signaling molecule that plays an important role in maintaining redox homeostasis [32]. The stimulation of inflammation can promote NOX (NADPH oxidase) assembly to catalyze extracellular $H_2O_2$ generation. Many aquaporins could transport $H_2O_2$ [33,34]. We found that *Aqp9* knockout reversed the LPS induced $H_2O_2$ accumulation in ET (Fig. 2I). This indicated that even though other transport channels may exist in ET, they were not sufficient to compensate for the inhibition of $H_2O_2$ transmembrane movement by AQP9 deficiency. It suggests that the transmembrane movement of $H_2O_2$ regulated by AQP9 plays an important role in ET inflammation.

We found that AQP9 promoted ET inflammation by inhibiting PP2A/NF-κB pathway. PP2A protein was decreased after LPS stimulation in WT mice, phosphorylated P65 of NF-κB pathway were increased (Fig. 3). PP2A is a holoenzyme composed of three subunits: a structure subunit (PP2A-A), a regulatory subunit (PP2A-B), and a catalytic subunit (PP2A-C). PP2A-A and PP2A-C dimers can bind to different regulatory subunits to form different PP2A heterotrimer [16]. PP2A binds to IKK complex and IκB-α, where it dephosphorylates key residues required for their activation and proteolytic processing, thereby suppressing NF-κB signaling [33]. By regulating the protein phosphorylation modification of kinases and phosphatases in the NF-κB and MAPK signaling pathways, it regulates various diseases such as cancer, neurodegenerative diseases and chronic inflammatory diseases and participates in the process of disease [29]. We found that AQP9 promotes $H_2O_2$ accumulation in ET, and that $H_2O_2$ could act as an intracellular signaling molecule to inhibit

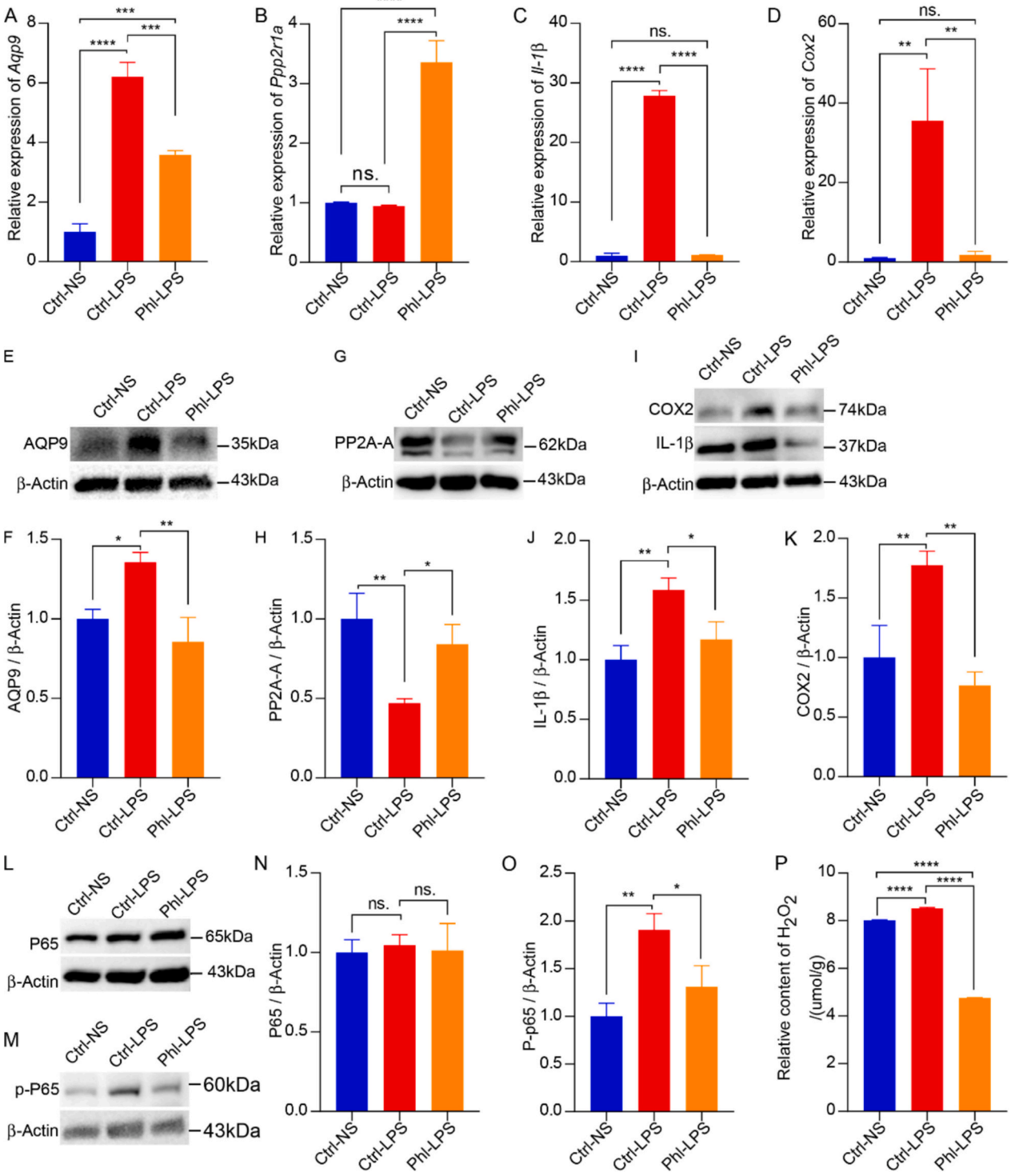


**Fig. 6.** Phloretin inhibited AQP9 expression and alleviated LPS-induced ET inflammation. A, *Aqp9* levels. Phloretin downregulated *Aqp9* expression. B-D, mRNA level of inflammatory factors. Phloretin upregulated *Ppp2r1a* and reduced *Il-1β*/*Cox2* mRNA levels. E-F. AQP9 levels. Phloretin reduced AQP9. G-K Inflammatory factor levels. Phloretin increased PP2A-A and decreased COX2/IL-1β levels. L-O, Effect of Phloretin on NF-κB activation. Phloretin inhibited phospho-P65 levels. P, $H_2O_2$ quantification. Phloretin suppressed LPS-induced $H_2O_2$ accumulation.

PP2A. In this study, $H_2O_2$ accumulation in ET inhibited the expression of PP2A scaffold subunit. Its inhibitory effect on NF-κB signaling pathway is reduced, promoting ET inflammation.

We found that AQP9 expression was increased in ET inflammation induced by LPS intratympanic injection, and *Aqp9* knockout could significantly inhibit LPS-induced ET inflammation. Therefore, exploring effective methods to inhibit AQP9 expression has potential clinical value. AQPs inhibitors include metal-related inhibitors, quaternary ammonium salts, and small molecule inhibitor. HTS13286 affects the water and solute permeability of AQP9, resulting in decreased glycerol-dependent glucose production. However, the amount of HTS13286 required to inhibit glycerol gluconeogenesis is high, which is not suitable for the experiments in vivo [34]. RG100204 inhibited AQP9 expression in taurocholate-induced acute pancreatitis and acute

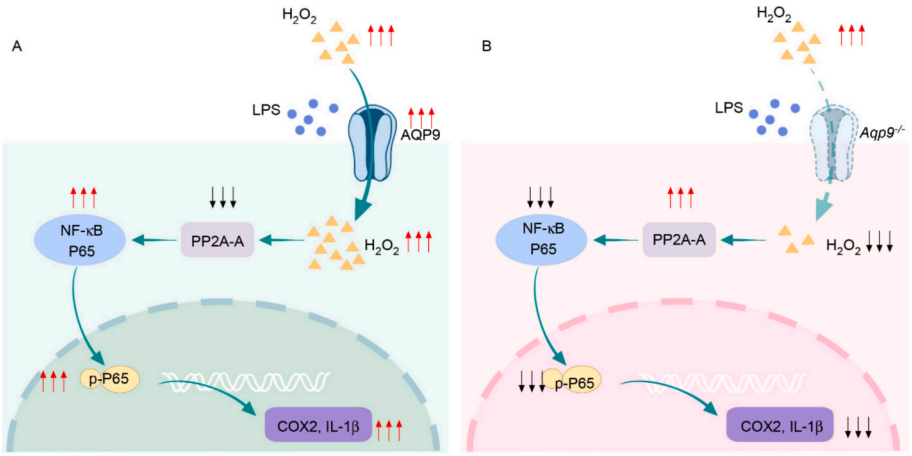


**Fig. 7.** AQP9 promotes LPS-induced ET inflammation via the PP2A/NF-κB pathway. A. Wild-type mechanism: LPS stimulation increases AQP9-mediated $H_2O_2$ transport, leading to intracellular $H_2O_2$ accumulation. Elevated $H_2O_2$ inhibits PP2A-A activity, thereby relieving its suppression of P65 phosphorylation and activating NF-κB–driven inflammatory responses. B. *Aqp9* knockout phenotype: Loss of AQP9 reduces $H_2O_2$ accumulation, restores PP2A-A activity, enhances its inhibition of P65 phosphorylation, and consequently blocks NF-κB activation and downstream inflammation.

pancreatitis induced by cauropsin and LPS. It reduced cell apoptosis, NF-κB signaling, and NLRP3 expression in the lungs of acute pancreatitis animals. At the same time, it significantly increased the Nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent anti-oxidative stress response [35]. Hawthorn soluble dietary fiber can express a variety of aquaporins, promote intestinal peristalsis, and relieve constipation in mice [36]. Inhibition of AQP9 by phloretin reduced proinflammatory cytokine production and lung injury in sepsis [11]. Compared with AQP9 specific inhibition of HTS13286 and RG100204, phloretin is not a specific AQP9 inhibitor, but it has been applied in clinical practice, which is conductive to clinical translation. In this study, phloretin can effectively inhibit LPS-induced expression of AQP9 and reduce the expression of inflammatory factors in ET, suggesting its clinical transformation potential.

In this study, an acute ET inflammation model was constructed by LPS injection for 48 h and did not address the role of AQP9 in chronic inflammation. AQP9 can activate dendritic cells and macrophages in chronic inflammatory diseases such as psoriasis, and Crohn's disease [31,37]. Therefore, exploring the role of AQP9 in the chronic ET inflammation may help the treatment of chronic otitis media. In addition, phloretin was intraperitoneally injected twice a week for 6 consecutive weeks, and phloretin was not a specific inhibitor of AQP9. Optimizing phloretin reagent or using AQP9 specific inhibitors is conducive to promoting the clinical transformation of AQP9 targeted therapy for ET inflammation in the future. In addition, this study limited in animal models, and further studies are needed to validate these findings in human models.

## 5. Conclusion

In summary, LPS promotes the expression of AQP9 in ET, leading to $H_2O_2$ accumulation (Fig. 7). The elevated $H_2O_2$ inhibited the PP2A-A and reduced its inhibitory effect on P65 phosphorylation and nuclear translocation, and subsequently activated NF-κB signaling pathway, which accelerates the ET inflammation. *Aqp9* knockout or treatment with phloretin significantly attenuates LPS-induced ET inflammation. Therefore, AQP9 might be a novel therapeutic target for the ET inflammation.

## CRediT authorship contribution statement

**Rui Li:** Visualization, Conceptualization, Writing – original draft. **Xuan Fang:** Visualization, Validation. **Yueqi Wang:** Visualization. **Zhen Zhong:** Project administration. **Junxiao Jia:** Methodology. **Yu Song:** Project administration. **Quancheng Cheng:** Investigation. **Man Li:** Investigation. **Chao Wang:** Methodology. **Weiguang Zhang:** Resources, Conceptualization. **Junxiu Liu:** Supervision, Funding acquisition, Conceptualization. **Huaicun Liu:** Writing – review & editing.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.145655.

## References

[1] S. Liu, X. Ni, J. Zhang, Assessment of the eustachian tube: a review, Eur. Arch. Otorhinolaryngol. 280 (9) (2023) 3915–3920, https://doi.org/10.1007/s00405-023-08026-5.
[2] A. Yang, M. Jv, J. Zhang, Y. Hu, J. Mi, H. Hong, Analysis of risk factors for otitis media with effusion in children with adenoid hypertrophy, Risk Manag. Healthc. Policy 16 (2023) 301–308, https://doi.org/10.2147/rmhp.S399499.
[3] T. Ockermann, U. Reineke, T. Upile, J. Ebmeyer, H.H. Sudhoff, Balloon dilatation eustachian tuboplasty: a clinical study, Laryngoscope 120 (7) (2010) 1411–1416, https://doi.org/10.1002/lary.20950.
[4] H. Oehlandt, J. Pulkkinen, L. Haavisto, Balloon dilation of the eustachian tube in chronic eustachian tube dysfunction: a retrospective study of 107 patients, J. Int. Adv. Otol. 18 (6) (2022) 495–500, https://doi.org/10.5152/iao.2022.21379.
[5] Sarah Alshehri, Abdullah Musleh, The role of eustachian tube dysfunction in recurrent chronic otitis media: a cross-sectional study of anatomical and functional variations, Healthcare (Basel) 13 (1) (2025) 77, https://doi.org/10.3390/healthcare13010077.

[6] X. Yu, H. Zhang, S. Zong, H. Xiao, Allergy in pathogenesis of eustachian tube dysfunction, World Allergy Organ J. 17 (1) (2024) 100860, https://doi.org/10.1016/j.waojou.2023.100860.
[7] C.S. Moon, D. Moon, S.K. Kang, Aquaporins in cancer biology, Front. Oncol. 12 (2022) 782829, https://doi.org/10.3389/fonc.2022.782829.
[8] I.V. da Silva, S. Garra, G. Calamita, G. Soveral, The multifaceted role of aquaporin-9 in health and its potential as a clinical biomarker, Biomolecules 12 (7) (2022) 897, https://doi.org/10.3390/biom12070897.
[9] S. De Santis, G. Serino, M.R. Fiorentino, V. Galleggiante, P. Gena, G. Verna, M. Liso, M. Massaro, J. Lan, J. Troisi, I. Cataldo, A. Bertamino, A. Pinto, P. Campiglia, A. Santino, G. Giannelli, A. Fasano, G. Calamita, M. Chieppa, Corrigendum: Aquaporin-9 contributes to the maturation process and inflammatory cytokine secretion of murine dendritic cells, Front. Immunol. 10 (2019) 216, https://doi.org/10.3389/fimmu.2019.00216.
[10] A. Tesse, P. Gena, M. Rützler, G. Calamita, Ablation of aquaporin-9 ameliorates the systemic inflammatory response of lps-induced endotoxic shock in mouse, Cells 10 (2) (2021) 435, https://doi.org/10.3390/cells10020435.
[11] P. Liang, M. Zhu, X. Sun, L. Wang, B. Li, S. Ming, M. Younis, J. Yang, Y. Wu, X. Huang, Lncrna-mrna co-expression analysis reveals aquaporin-9-promoted neutrophil extracellular trap formation and inflammatory activation in sepsis, Int. Immunopharmacol. 140 (2024) 112916, https://doi.org/10.1016/j.intimp.2024.112916.
[12] Y.M. Chun, K.H. Chang, D.J. Lim, Expressions of aquaporin family of water channel proteins in rat tubotympanum in vivo and in vitro [M], in: D.J. Lim, C.D. Bluestone, M. Casselbrant (Eds.), Proceedings of the 7th International Symposium on Recent Advances in Otitis Media, 1999 (Fort Lauderdale, FL, USA).
[13] J.H. Ahn, M.G. Cho, S. Sohn, J.H. Lee, Inhibition of pp2a activity by h(2)o(2) during mitosis disrupts nuclear envelope reassembly and alters nuclear shape, Exp. Mol. Med. 51 (6) (2019) 1–18, https://doi.org/10.1038/s12276-019-0260-0.
[14] D. Fan, Y. Zhong, Y. Dong, M. Du, K. Huang, X. Li, Transducin-like enhancer of split 3 protects against lipopolysaccharide-induced inflammation through dead-box helicase 5-activating transcription factor 1-protein phosphatase 2 regulatory subunit 5a signaling, J. Adv. Res. (2025), https://doi.org/10.1016/j.jare.2025.03.041 (S2090-1232(2025)00200-00200).
[15] D.A. Neale, J.C. Morris, N.M. Verrills, A.J. Ammit, Understanding the regulatory landscape of protein phosphatase 2a (pp2a): pharmacological modulators and potential therapeutics, Pharmacol. Ther. 269 (2025) 108834, https://doi.org/10.1016/j.pharmthera.2025.108834.
[16] J. Pan, L. Zhou, C. Zhang, Q. Xu, Y. Sun, Targeting protein phosphatases for the treatment of inflammation-related diseases: from signaling to therapy, Signal Transduct. Target. Ther. 7 (1) (2022) 177, https://doi.org/10.1038/s41392-022-01038-3.
[17] Q. Cheng, H. Ding, J. Fang, X. Fang, H. Liu, J. Wang, C. Chen, W. Zhang, Aquaporin 9 represents a novel target of chronic liver injury that may antagonize its progression by reducing lipotoxicity, Oxidative Med. Cell. Longev. 2021 (2021) 5653700, https://doi.org/10.1155/2021/5653700.
[18] Q. Cheng, J. Zhang, H. Ding, Z. Wang, J. Fang, X. Fang, M. Li, R. Li, J. Meng, H. Liu, X. Lu, Y. Xu, C. Chen, W. Zhang, Integrated multiomics analysis reveals changes in liver physiological function in aqp9 gene knockout mice, Int. J. Biol. Macromol. 245 (2023) 125459, https://doi.org/10.1016/j.ijbiomac.2023.125459.
[19] J.S. Han, Y.L. Kim, H.J. Yu, J.M. Park, Y. Kim, S.Y. Park, S.N. Park, Safety and efficacy of intratympanic alpha-lipoic acid injection in a mouse model of noise-induced hearing loss, Antioxidants (Basel) 11 (8) (2022) 1423, https://doi.org/10.3390/antiox11081423.
[20] Y. Chai, W. He, W. Yang, A.P. Hetrick, J.G. Gonzalez, L. Sargsyan, H. Wu, T.T. K. Jung, H. Li, Intratympanic lipopolysaccharide elevates systemic fluorescent gentamicin uptake in the cochlea, Laryngoscope 131 (9) (2021) E2573–e2582, https://doi.org/10.1002/lary.29610.
[21] C. Nie, Y. Zou, S. Liao, Q. Gao, Q. Li, Molecular targets and mechanisms of 6,7-dihydroxy-2,4-dimethoxyphenanthrene from chinese yam modulating nf-κb/cox-2 signaling pathway: the application of molecular docking and gene silencing, Nutrients 15 (4) (2023) 883, https://doi.org/10.3390/nu15040883.
[22] Y.H. Yee, S.J.F. Chong, L.R. Kong, B.C. Goh, S. Pervaiz, Sustained ikkβ phosphorylation and nf-κb activation by superoxide-induced peroxynitrite-mediated nitrotyrosine modification of b56γ3 and pp2a inactivation, Redox Biol. 41 (2021) 101834, https://doi.org/10.1016/j.redox.2020.101834.
[23] G. Iannella, G. Magliulo, J.R. Lechien, A. Maniaci, T. Perrone, P.C. Frasconi, A. De Vito, C. Martone, S. Ferlito, S. Cocuzza, G. Cammaroto, G. Meccariello, V. Monticone, A. Greco, M. de Vincentiis, M. Ralli, V. Savastano, S. Bertin, A. Pace, A. Milani, R. Polimeni, S. Pelucchi, A. Ciorba, C. Vicini, Impact of covid-19 pandemic on the incidence of otitis media with effusion in adults and children: a multicenter study, Eur. Arch. Otorhinolaryngol. 279 (5) (2022) 2383–2389, https://doi.org/10.1007/s00405-021-06958-4.
[24] Kai-Shan Yang, Wei-Chih Chen, Ching-Nung Wu, Yin-Shen Wee, Ching-Shuen Wang, Cheng-Chih Wu, Sheng-Dean Luo, Endoscopic sinus surgery significantly reduces eustachian tube dysfunction symptoms in patients with chronic rhinosinusitis: a systematic review and meta-analysis, Biomedicines 12 (11) (2024) 2848, https://doi.org/10.3390/biomedicines12112484.
[25] H.M. Juszczak, P.A. Loftus, Role of allergy in eustachian tube dysfunction, Curr Allergy Asthma Rep 20 (10) (2020) 54, https://doi.org/10.1007/s11882-020-00951-3.
[26] A. Matsushima, H. Ogura, T. Koh, T. Shimazu, H. Sugimoto, Enhanced expression of aquaporin 9 in activated polymorphonuclear leukocytes in patients with systemic inflammatory response syndrome, Shock 42 (4) (2014) 322–326, https://doi.org/10.1097/shk.0000000000000218.
[27] Y.C. Chuang, S.Y. Wu, Y.C. Huang, C.K. Peng, S.E. Tang, K.L. Huang, Cell volume restriction by mercury chloride reduces m1-like inflammatory response of bone marrow-derived macrophages, Front. Pharmacol. 13 (2022) 1074986, https://doi.org/10.3389/fphar.2022.1074986.
[28] J. Jing, J. Sun, Y. Wu, N. Zhang, C. Liu, S. Chen, W. Li, C. Hong, B. Xu, M. Chen, Aqp9 is a prognostic factor for kidney cancer and a promising indicator for m2 tam polarization and cd8+ t-cell recruitment, Front. Oncol. 11 (2021) 770565, https://doi.org/10.3389/fonc.2021.770565.
[29] Y. Li, X. Yang, X. Li, S. Wang, P. Chen, T. Ma, B. Zhang, Astragaloside iv and cycloastragenol promote liver regeneration through regulation of hepatic oxidative homeostasis and glucose/lipid metabolism, Phytomedicine 135 (2024) 156165, https://doi.org/10.1016/j.phymed.2024.156165.
[30] Y. Li, X. Yang, T. Bao, X. Sun, X. Li, H. Zhu, B. Zhang, T. Ma, Radix astragali decoction improves liver regeneration by upregulating hepatic expression of aquaporin-9, Phytomedicine 122 (2024) 155166, https://doi.org/10.1016/j.phymed.2023.155166.
[31] Q.Q. Zhu, Y. Zhang, L. Cui, L. Ma, K.W. Sun, Downregulation of aqp9 ameliorates nlrp3 inflammasome-dependent inflammation and pyroptosis in crohn's disease by inhibiting the p38 mapk signaling pathway, Mol. Biotechnol. (2025), https://doi.org/10.1007/s12033-025-01382-z.
[32] R.R. Manoharan, A. Prasad, P. Pospíšil, J. Kzhyshkowska, Ros signaling in innate immunity via oxidative protein modifications, Front. Immunol. 15 (2024) 1359600, https://doi.org/10.3389/fimmu.2024.1359600.
[33] A.R. Clark, M. Ohlmeyer, Protein phosphatase 2a as a therapeutic target in inflammation and neurodegeneration, Pharmacol. Ther. 201 (2019) 181–201, https://doi.org/10.1016/j.pharmthera.2019.05.016.
[34] S. Jelen, S. Wacker, C. Aponte-Santamaría, M. Skott, A. Rojek, U. Johanson, P. Kjellbom, S. Nielsen, B.L. de Groot, M. Rützler, Aquaporin-9 protein is the primary route of hepatocyte glycerol uptake for glycerol gluconeogenesis in mice, J. Biol. Chem. 286 (52) (2011) 44319–44325, https://doi.org/10.1074/jbc.M111.297002.
[35] J. Chen, X. Zhu, Z. Wang, M. Rützler, Q. Lu, H. Xu, R. Andersson, Y. Dai, Z. Shen, G. Calamita, S. Xie, Y. Bai, B. Chen, Inhibition of aquaporin-9 ameliorates severe acute pancreatitis and associated lung injury by nlrp3 and nrf2/ho-1 pathways, Int. Immunopharmacol. 137 (2024) 112450, https://doi.org/10.1016/j.intimp.2024.112450.
[36] H. Zhang, Q. Zu, J. Zhang, S. Liu, G. Zhang, X. Chang, X. Li, Soluble dietary fiber of hawthorn relieves constipation induced by loperamide hydrochloride by improving intestinal flora and inflammation, thereby regulating the aquaporin ion pathway in mice, Foods 13 (14) (2024) 2220, https://doi.org/10.3390/foods13142220.
[37] Z.A. Chen, H.H. Ma, Y. Wang, H. Tian, J.W. Mi, D.M. Yao, C.J. Yang, Integrated multiple microarray studies by robust rank aggregation to identify immune-associated biomarkers in crohn's disease based on three machine learning methods, Sci. Rep. 13 (1) (2023) 2694, https://doi.org/10.1038/s41598-022-26345-1.