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Effects of polysaccharides on turbot *Scophthalmus maximus*: evaluation with a head kidney macrophage cellular model

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ABSTRACT: Polysaccharides exhibit a multitude of biological activities, including antioxidant, antitumor, immunoregulatory, hepatoprotective, and anti-inflammatory effects, but it is not known whether such effects occur in fish. Head kidney macrophages from turbot *Scophthalmus maximus* L. were isolated and cultured to examine the responsiveness to natural polysaccharides as potential immune stimulators. Polysaccharides used in the research included *Echinacea purpurea* polysaccharide (EPP), *Astragalus* polysaccharide (APS), lentinan (LNT), seaweed polysaccharide (SPS), and laminarin (LAM). The test compounds were added to the cultures and assessed for their effects on the growth and immunomodulatory functions of the cells. Based on the results of cell activity, reactive oxygen species, and nitic oxide assays, APS was selected as an immune stimulator. After addition of APS to the culture medium, a comprehensive proteomic analysis was conducted to identify signaling pathways responsible for the immune effects on macrophages. Specific immune pathway proteins were upregulated in cells in response to the addition of APS, including macrophage migration inhibitory factor, myosin-α, metalloproteinase inhibitor, and collagenase type III. In particular, compared with non-stimulated cells, the expression level of the TLR22 receptor was significantly increased in stimulated macrophages ($p < 0.01$). A KEGG pathway analysis indicated that relevant pathways were activated, including TNF, PI3K-Akt, and NF-κB signaling pathways. ELISA and qRT-PCR analysis also indicated that APS reduced IL-1β and TNF-α levels in the cells following lipopolysaccharide (LPS) stimulation. These data suggest that APS produced an immunoprotective effect on the head kidney macrophages of turbot at 800 μg ml^{-1}, and enhanced cell proliferation. Our results provide evidence for anti-inflammatory properties of APS. As such, APS could be a candidate immunopotentiating agent for fish.

KEY WORDS: *Astragalus* polysaccharide · *Scophthalmus maximus* L. · Immune regulation

1. INTRODUCTION

Plant extracts have numerous beneficial regulatory functions in the body. In particular, the immunoregulatory effects of phytochemicals have been assessed for polyphenols, polysaccharides, flavonoids, and alkaloids (Yin et al. 2019, Siddiqui et al. 2020, Efferth & Oesch 2021). Polysaccharides can lower blood lipids (Cheng et al. 2020), function as anticoagulants (de Araujo et al. 2021), and act as anti-viral (Guo et al. 2021), anti-aging (Shen et al. 2017), hypoglycemic, and anti-tumor agents (He et al. 2019). Therefore, the utilization of natural substances presents a promising approach to supplement traditional therapies, laying a foundation for the application of polysaccharides in veterinary medicine.

Echinacea purpurea polysaccharide (EPP) have been demonstrated to alleviate lipopolysaccharide (LPS)-induced acute renal injury by inhibiting inflammation and mitogen-activated protein kinase (MAPK) signaling, providing a potential therapeutic avenue for acute renal injury (Shi et al. 2021). EPP may also inhibit inflammation and apoptosis, and activate Toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) signaling

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to alleviate LPS-induced lung injury (B. Zhang et al. 2020). Notably, there is substantial evidence supporting the immunomodulatory effects of EPP (Pillai et al. 2007). *Astragalus* polysaccharide (APS), derived from *Astragalus membranaceus*, exhibits antioxidant, anti-inflammatory, antibacterial, and antiviral activities, directly influencing the immune system (Zhou et al. 2017, Liao et al. 2021). Studies have shown that APS enhances immune function in fish (Feng et al. 2021, Liu et al. 2022) and upregulates the expression of Toll-like receptors (TLRs) in turbot, indicating its potential immunomodulatory role (Sun et al. 2020). Lentinan (LNT), extracted from *Lentinus edodes*, restores immune-related gene expression in immunocompromised mice and alleviates diarrhea symptoms in piglets infected with rotavirus, highlighting its therapeutic potential (Luo et al. 2018, Fan et al. 2021). Seaweed polysaccharide (SPS), derived from brown algae and marine plants, exert positive effects on intestinal metabolic regulation and immune function activation (Balboa et al. 2013, Zhu et al. 2019, Cheng et al. 2020, Guo et al. 2021, Venardou et al. 2021). Additionally, laminarin (LAM), a marine polysaccharide, modulates the intestinal flora of broiler chickens and enhances immune responses in mice (Zhu et al. 2019, Venardou et al. 2021), although it is not known whether such effects also occur in fish.

Turbot *Scophthalmus maximus L.* is a significant species in marine aquaculture, with substantial global production. However, disease outbreaks pose significant challenges to the sustainability of the industry. Enhancing growth rates and disease resistance has thus become a primary focus of breeding programs (Ma et al. 2021).

Non-specific immunity plays a crucial role in fish defense mechanisms, with the head kidney serving as a key immune organ sensitive to environmental changes (Geven & Klaren 2017, Bjørgen & Koppang 2021). Macrophages, primary immune cells in fish, produce various cytokines and reactive oxygen species (ROS) in response to pathogens, making them vital indicators of immune function (Chiu & Lin 2008, Yang et al. 2020).

Polysaccharides can improve the phagocytic function of macrophages, increase cytokine production, and enhance immunity (Luo et al. 2024, Pei et al. 2024, Sun et al. 2024, Xue et al. 2024), often by stimulating TLRs on macrophages (Hansen et al. 2011). While TLR4 and TLR2 are prominent in mammals, the corresponding receptors in fish remain unidentified.

This study investigated the immunomodulatory effects of polysaccharides on turbot head kidney macrophages, focusing on 5 polysaccharides and their impact on immune function. APS emerged as the most promising candidate, prompting further investigation into its mechanism of action through proteomic and molecular analyses.

2. MATERIALS AND METHODS

2.1. Isolation and identification of cells

LNT, EPP, APS, SPS, and LAM were obtained from Sigma-Aldrich (purity >90%). Turbot *Scophthalmus maximus* L. (Haiyang Huanghai Fisheries) was used for cell preparation, and healthy individuals aged 6 to 7 mo were selected for the study. The head kidneys of the anesthetized turbot were removed aseptically (ether concentration 10 ml l^{-1}). The cells were then separated by gross dissection and subjected to a 34 to 51% Percoll gradient by centrifugation using standard techniques (Mills et al. 2017). The macrophage cells were cultured in L-15 medium (Gibco) supplemented with fetal bovine serum and triple antibody solution as previously described.

2.2. Effects of polysaccharides on macrophage function

The MTT assay kit (Beijing Solarbio Science) was used to detect cell proliferation activity. In brief, the polysaccharides LNT, EPP, APS, SPS, and LAM (at concentrations of 1000, 800, 500, 250, 125, 62.5, 31, and 15.5 μg ml^{-1}) were added to macrophage cultures (5×10^6 cells ml^{-1}) and then incubated at 18°C for 24 h (Luo et al. 2024). The cells were then centrifuged at 400 × *g* for 5 min, and the cell supernatants were added to 96-well plates for the MTT assay, following the instructions provided by the manufacturer. Nitric oxide (NO) and ROS production of the macrophages were measured using DAF-FM diacetate and the Reactive Oxygen Species Assay Kit (Beyotime Biotechnology). All experiments were repeated 3 times.

2.3. Proteomic analysis

Proteomic analysis was conducted using the tandem mass tags (TMT) method for *in vitro* labeling and quantitation, and was performed commercially (PTM Biolabs, Hangzhou). Head kidney macrophages were stimulated with 800 μg ml^{-1} APS for 24 h, and then total protein extracts were generated using a commercial kit (Beijing ComWin Biotech) and used for

proteomic analysis. Non-stimulated cells were used to provide a negative control.

2.4. Expression analyses

ELISA assay kits (Shanghai Enzyme-linked Biotechnology) were used to measure tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10, and transforming growth factor (TGF)-β cytokine levels in macrophage cell culture supernatants. Macrophages were divided into a normal cell control group, an LPS (2 μg ml^{-1}) stimulation group, and an LPS+APS experimental group. Quantitative real-time PCR was used to measure steady-state mRNA levels in cultured macrophages. Total RNA (57 ng μl^{-1}) was isolated using TRIzol reagent and cDNA (430 ng μl^{-1}) was constructed using a commercial kit (Invitrogen). Concentrations of RNA and cDNA were determined by a microspectrophotometer (Thermo Scientific NanoDrop). Gene expression levels were measured by real-time detection on a QuantStudio 5 instrument (Applied Biosystems) using s SYBR green kit (Takara Biomedical Technology) and gene-specific PCR primers following the manufacturer's instructions. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). The primers and qPCR conditions are provided in Tables 1 & 2. The qPCR mixture was composed of 2× One-Step TB Green RT-PCR Buffer (10 μl), Prime Script 1 Step Enzyme Mix 2 (0.8 μl), forward and reverse primers at a concentration of 10 μM (0.8 μl), template (2 μl), and RNA-free H_2O (5.6 μl).

3. RESULTS

3.1. Macrophage isolation and culture

The cells actively proliferated and displayed robust growth in culture. Initially ($T = 0$), the cells were uniform and mostly round in appearance. At $T = 2$ d, the macrophages formed irregular shapes with numerous pseudopodia. Swiss-Giemsa staining of the cells revealed fusiform nuclei and the presence of many large pink and purple-blue granules in the cytoplasm, and the cell growth status was representative of healthy cells (Fig. 1).

3.2. Polysaccharide screening of macrophages

The presence of LNT in the cultures produced no cytotoxic effects on the cells when exposed for 24 and 48 h at levels of 62.5 to 250 μg ml^{-1}. EPP stimulated the macrophages and promoted proliferation after 24 h exposure, but was cytotoxic after 48 h. APS promoted proliferation at 800 μg ml^{-1} and cytotoxic effects were apparent at some concentrations after 48 h. SPS and LAM showed no cytotoxicity after 24 h and 125 and 250 μg ml^{-1} LAM promoted a moderate level of proliferation (Fig. 2A–E). The comprehensive effect of APS was significantly superior to that of the other 4 polysaccharides in promoting cell growth.

NO is an important effector molecule secreted by activated macrophages that has a non-specific immune cytotoxic effect. Our cultured cells produced NO following exposure to 250 and 500 μg ml^{-1} LNT, 62.5 to 1000 μg ml^{-1} EPP, 100 to 1000 μg ml^{-1} APS, 125 to 500 μg ml^{-1} SPS, and 250 to 1000 μg ml^{-1} LAM (Fig. 2F–J). Interestingly, addition of APS, EPP, and SPS to the cultures inhibited ROS production and thus exerted an anti-oxidative protective effect that was not apparent with LAM and LNT (Fig. 2K–O). Based on the above results, APS at a concentration of 800 μg ml^{-1} generated the optimal effect of promoting macrophage growth and effectively regulating the levels of NO and ROS production.

3.3. Proteomic analysis

APS was added to head kidney macrophages at 800 μg ml^{-1} and total protein extracts were gener-

Table 1. Oligonucleotide primer sets for real-time qPCR analyses of the steady state mRNA levels of the cytokines TNF-α, TGF-β, IL-1β, and IL-10, and the receptors TLR2, TLR3, TLR5a, TLR5b, and TLR22

Name	Primer sequence (5′→3′)
18S-F	CAC AGT GCC CAT CTA TGA G
18S-R	CCA TCT CCT GCT CGA AGT C
TLR2-F	AGG AGC CAA GGG AGA CCG AT
TLR2-R	GGC GCT CAT GAT GTT GTC C
TLR3-F	TGG TGT CGT CGA TTC AAA GC
TLR3-R	CCA ATC CAA CTA CTC CCC ACG
TLR22-F	ACA GAG ACT TCG AGC CAG GTA AAC CC
TLR22-R	CTT GTT CGG CAG TTT CCT CA
TNF-α-F	CCC TTA TCA TTA TGG CCC TT
TNF-α-R	TCC GAG TAC CGC CAT ATC CT
TGF-β-F	CTG CAG GAC TGG CTC AAA GG
TGF-β-R	CAT GGT CAG GAT GTA TGG TGG T
IL-1β-F	ATG GTG CGA TTT CTG TTC
IL-1β-R	CAC TTT GGG TCG TCT TTG
IL-10-F	AGT GAC GGA GGA CGC CAA GG
IL-10-R	ACA TCT GCT GAC ATC GGA CTT GAG
TLR5a-F	AGT CTC TTT GGT CTC AGG GC
TLR5a-R	TTT GGG TAA GAC ATC GGG CT
TLR5b-F	AAC AAC TTC CTA GCC TCC CC
TLR5b-R	CAT GTG AAA TCC TCC GCT GG

Table 2. qPCR conditions for the analysis of steady state mRNA levels of the cytokines TNF-α, TGF-β, IL-1β, and IL-10, and (B) the receptors TLR2, TLR3, TLR5a, TLR5b, and TLR22

Stage 1			Stage 2			Stage 3		
42°C	5 min	1 cycle	95°C	5 s	40 cycles	95°C	0 s	1 cycle
95°C	10 s		60°C	20 s		65°C	65°C	

ated from the cells after 24 h exposure. Proteome analysis indicated that most of the peptides were distributed in the size range of 7 to 20 amino acids, which is in line with the expected pattern based on enzymatic and mass spectrometry fragmentation modes. The distribution of peptide lengths identified by mass spectrometry met the quality control requirements (Fig. 3A). For biologically or technically duplicated samples, it is necessary to test whether the quantitative results of are statistically consistent. Pearson's correlation coefficient was used to assess repeatability (Fig. 3B), and it can be seen from this that both the APS group and the non-stimulated group had good reproducibility. We identified 58 upregulated and 148 downregulated proteins that accounted for 28.16 and 71.84% of the total, respectively (Fig. 3C,D).

The differentially expressed proteins of the APS-stimulated cells vs. non-stimulated cells indicate that APS induced the expression of significant immune regulatory pathways including NF-κB signaling and cytokine receptor pathways (Fig. 4A–E). We performed enrichment analysis at 3 levels (Gene Ontology [GO] classification, Kyoto Encyclopedia of Genes and Genomes [KEGG] pathways, and protein domains) for the differentially expressed proteins in each comparison group (using Fisher's exact test to calculate the p-value). Significant enrichment of functional types and pathways among the differentially expressed proteins ($p < 0.05$) is presented using bubble charts.

After the differentially expressed proteins in the test group were categorized according to GO classification, KEGG pathways, and protein structural domain enrichment, we further classified them into 4 groups based on the differentially expressed ploidy, referred to as Q1 to Q4. GO classification, KEGG pathway, and protein structural domain enrichment analyses were then performed for each of the Q groups separately, and clustering analyses were carried out to determine the correlation of protein functionality with differentially expressed ploidy in the comparison groups.

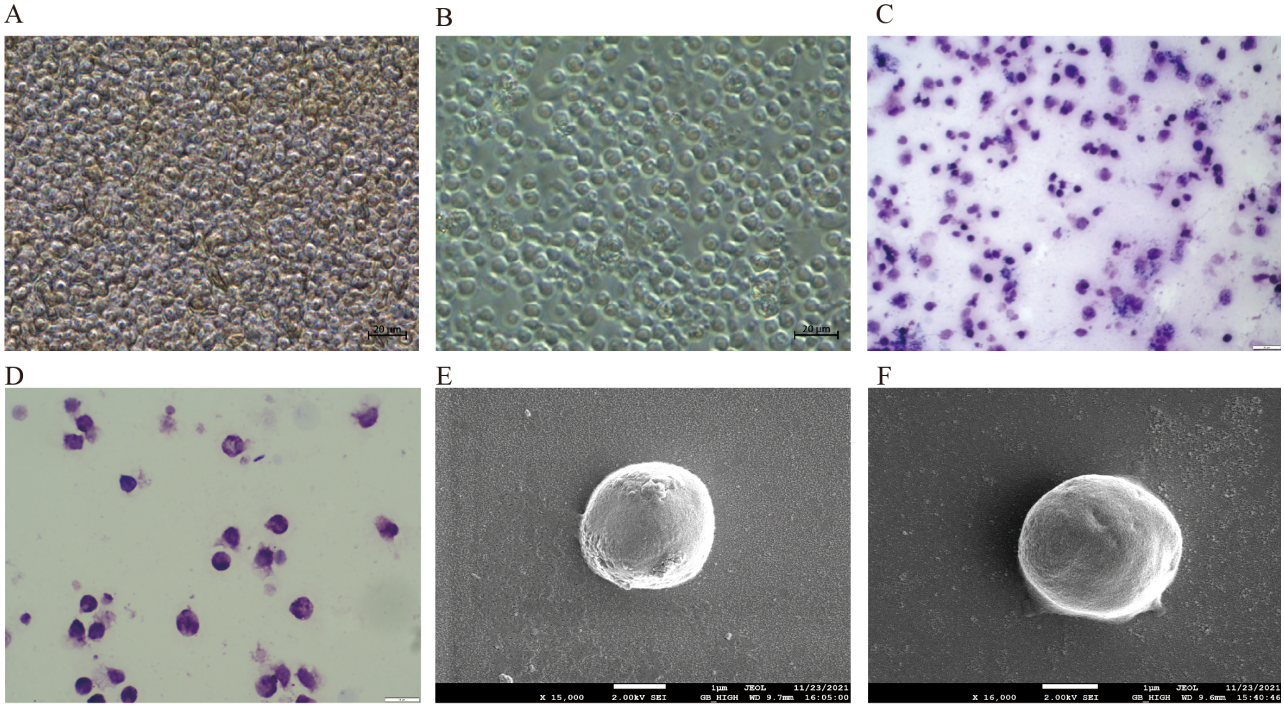

Fig. 1. Cell morphology. Macrophages derived from the turbot head kidney (A) at 0 h (initial plating) and (B) after 2 d in culture. (C,D) Swiss-Giemsa staining of the same cultures, respectively. Electron micrographs of macrophages at (E) 15 000× and (F) 16 000× magnification

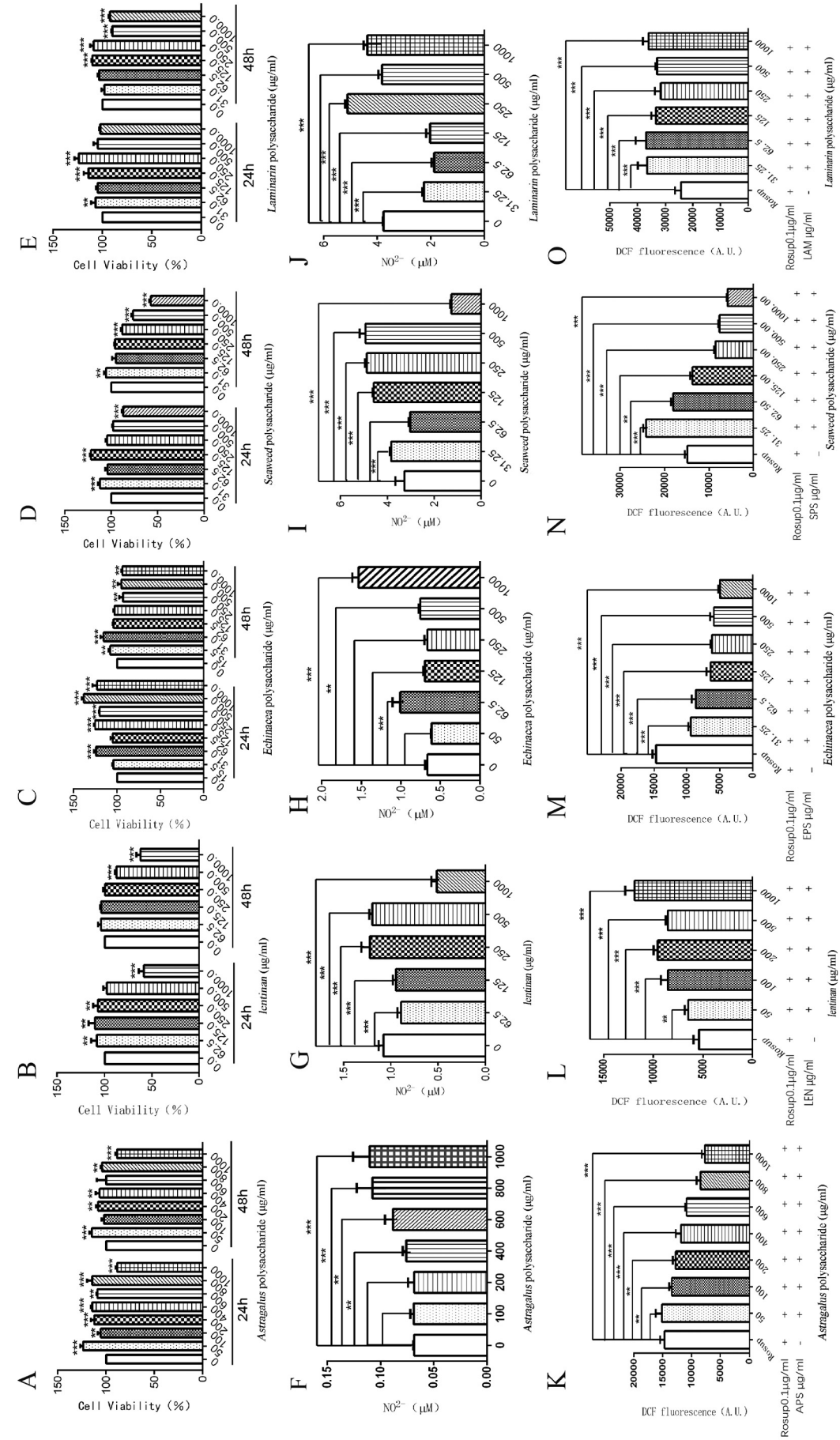

Fig. 2. Effects of polysaccharides on turbot head kidney macrophages. Initial screening of 5 polysaccharide types. (A–E) Macrophage proliferation, (F–J) NO production, and (K–O) reactive oxygen species (ROS) production. $^{**}p < 0.05$; $^{***}p < 0.01$

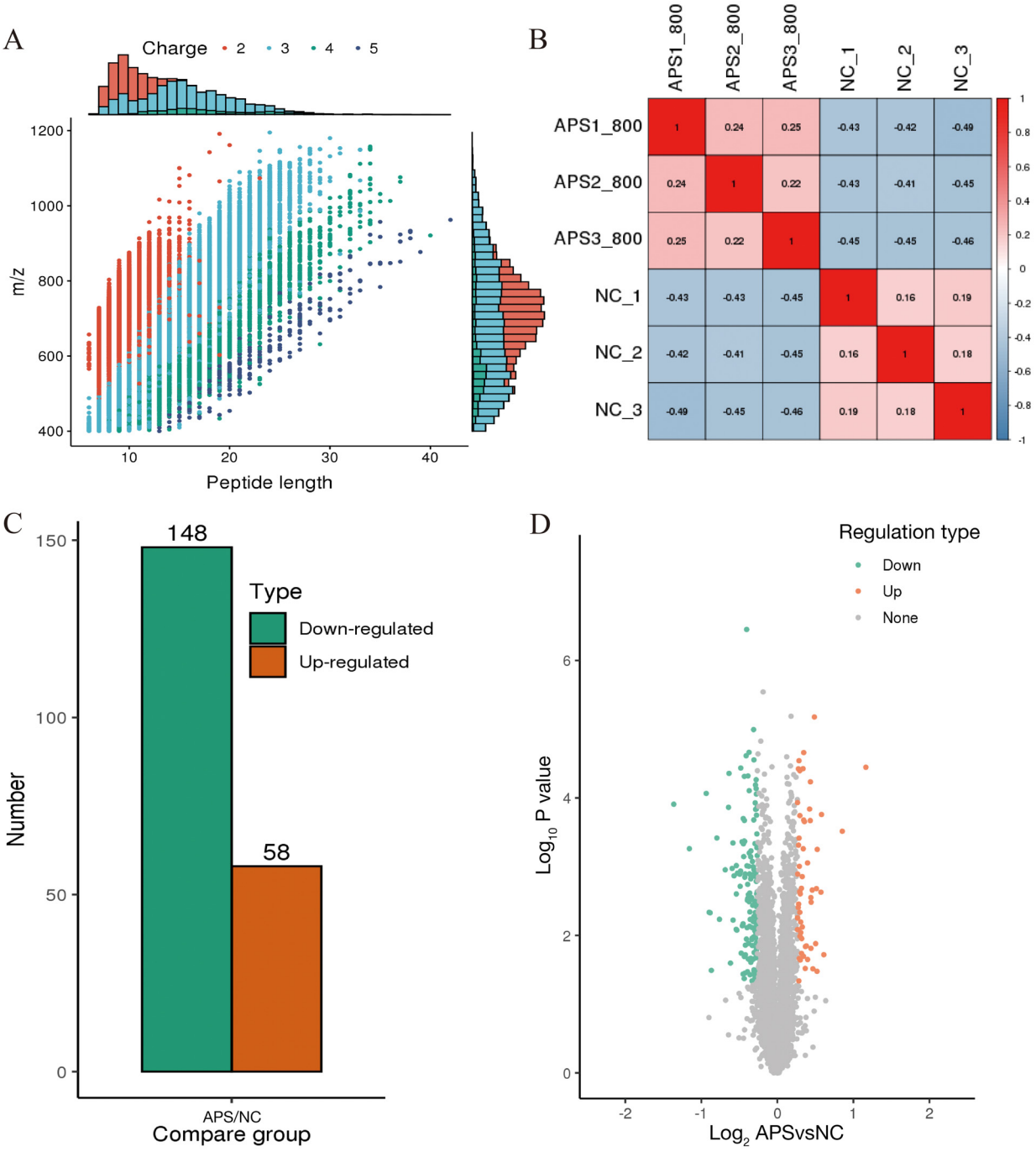

Fig. 3. Proteomic comparisons for *Astragalus* polysaccharide (APS)-stimulated (800 µg ml^{-1}) and non-stimulated (NC) turbot head kidney macrophage cell culture populations. (A) Peptide length distribution. (B) Pearson's correlation coefficient. Values close to −1 indicate negative correlation, those close to 1 indicate positive correlation, and those close to 0 indicate no correlation. The intragroup replicates are represented by 1, 2, and 3. (C,D) Differential expression of proteins by macrophages

We found that APS induced changes in the transduction of several biological processes and signaling pathways in macrophages, such as the cellular ROS response, cytokine-cytokine receptor interactions, cellular adhesion factor receptors, and the NF-κB, PI3K/AKT, and TNF signaling pathways (Fig. 5).

3.4. Cytokine and receptor expression following APS stimulation

The combined results of the proteomic data were used as a guide to screen for cytokine expression for further verification at the protein expression and

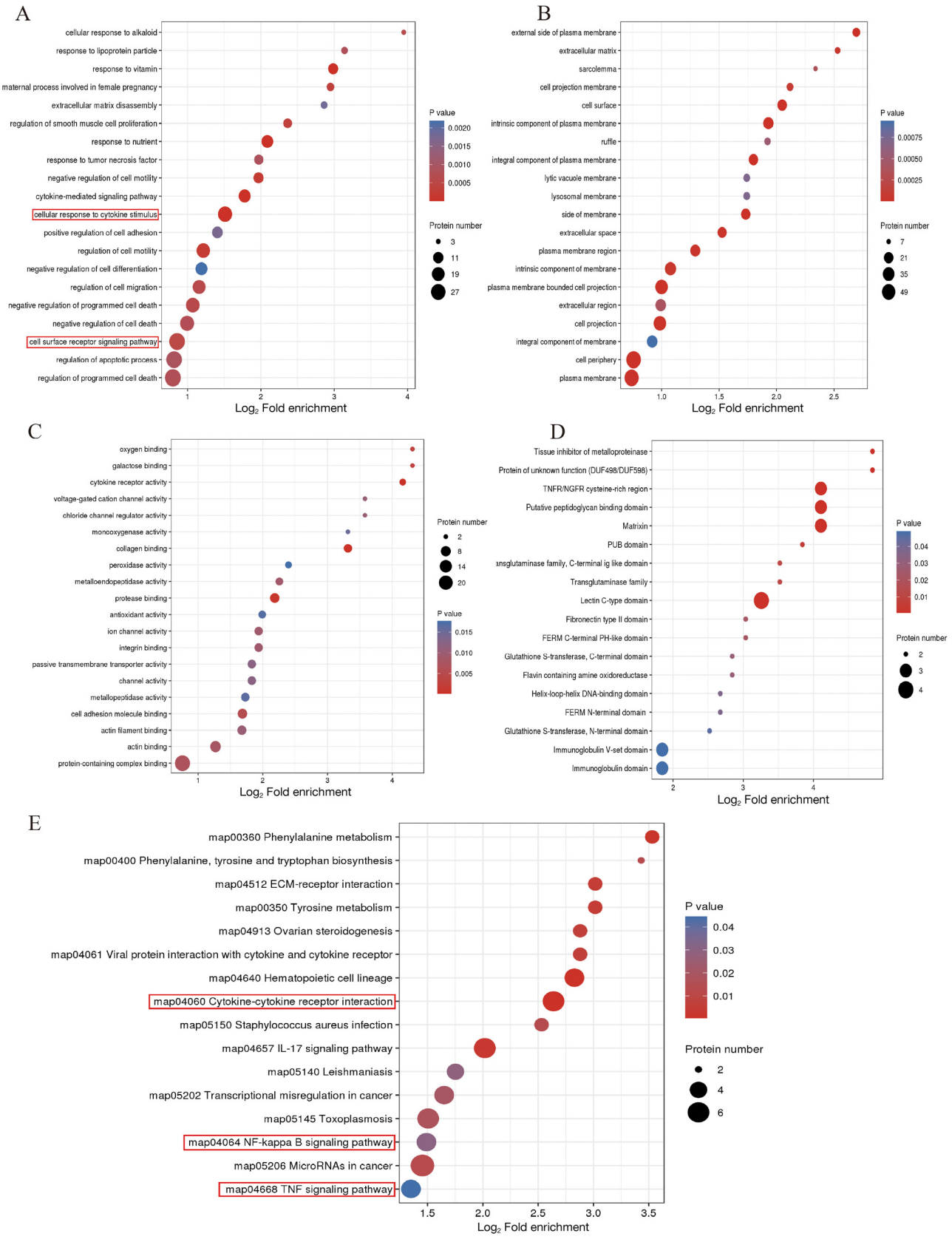

Fig. 4. KEGG pathway analysis of differentially expressed proteins in cultured turbot macrophages. (A) Biological process, (B) cellular component, (C) molecular function, (D) all differentially expressed proteins, and (E) protein domains. The bubble chart shows the top 20 categories with the most significant enrichment, with the *y*-axis representing functional classifications or pathways (red boxes indicate those related to immunity) and the *x*-axis representing the $\log_2$-transformed fold enrichment of the proportion of proteins allocated to the functional type

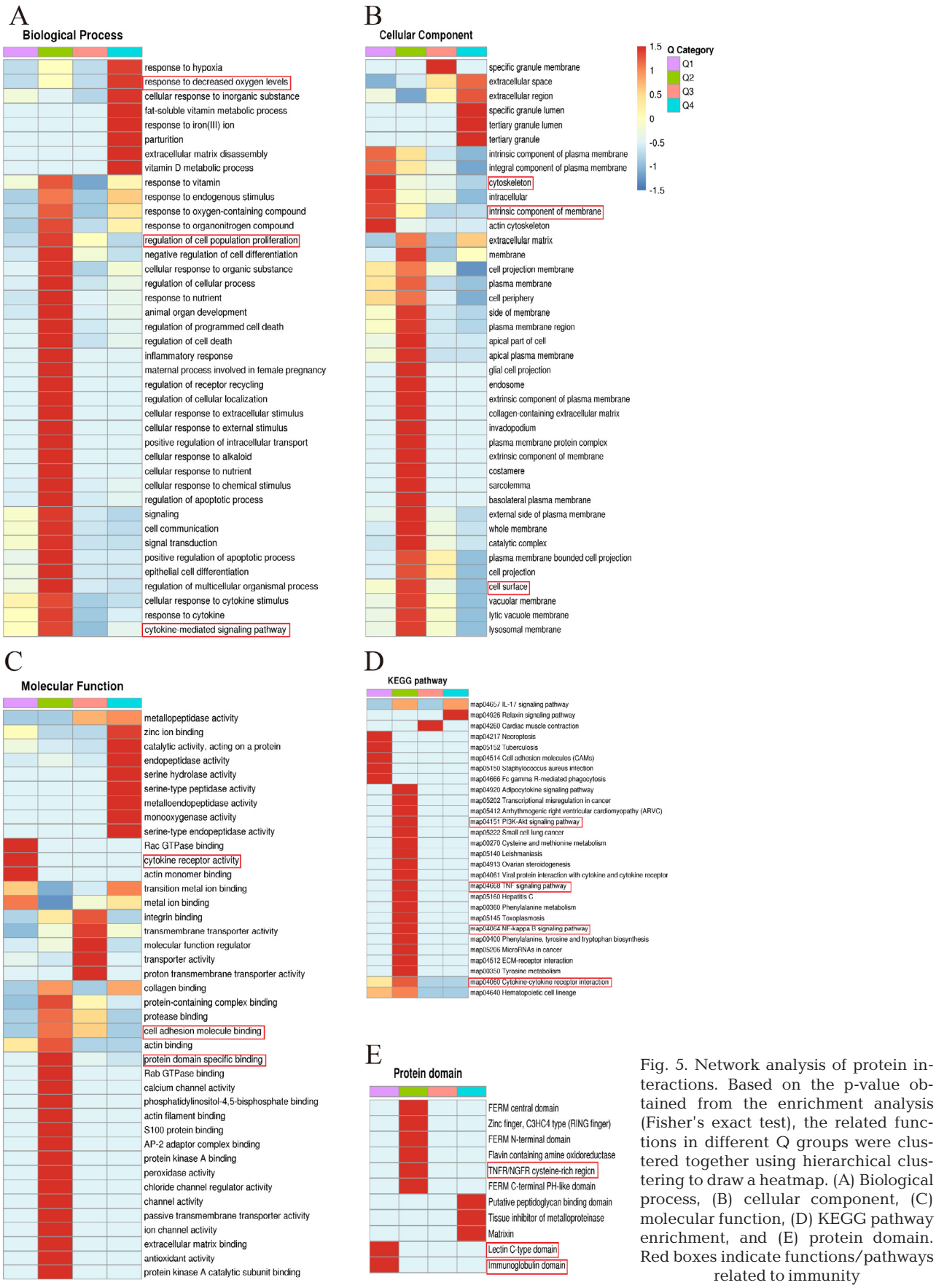

Fig. 5. Network analysis of protein interactions. Based on the p-value obtained from the enrichment analysis (Fisher's exact test), the related functions in different Q groups were clustered together using hierarchical clustering to draw a heatmap. (A) Biological process, (B) cellular component, (C) molecular function, (D) KEGG pathway enrichment, and (E) protein domain. Red boxes indicate functions/pathways related to immunity

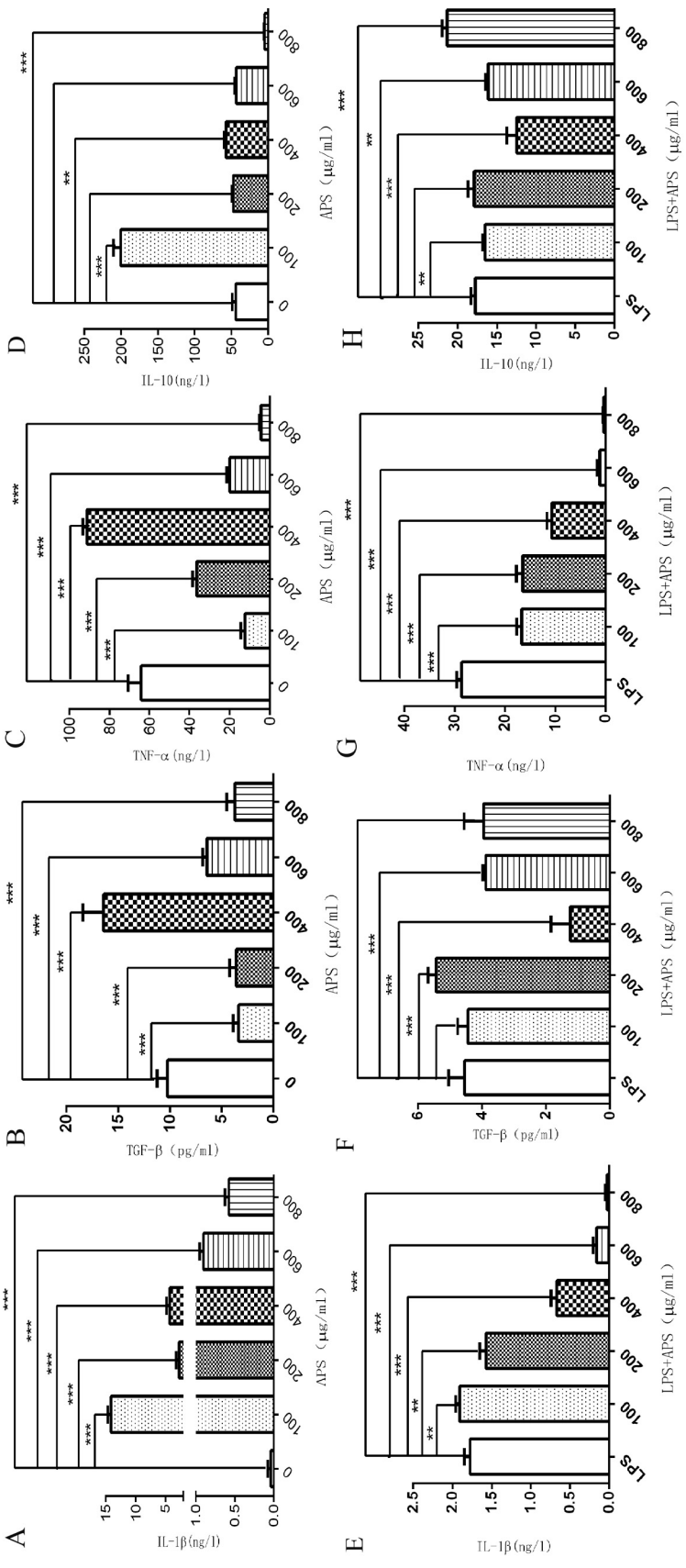

Fig. 6. Effects of APS stimulation of turbot head kidney macrophages on the expression of (A) IL-1β, (B) TGF-β, (C) TNF-α, and (D) IL-10. Effects of APS following LPS stimulation of macrophages on (E) IL-1β, (F) TGF-β, (G) TNF-α, and (H) IL-10. $^{**}p < 0.05$; $^{***}p < 0.01$

gene levels. The inflammatory cytokines TNF-α, TGF-β, IL-1β, and IL-10 were measured using ELISA assays.

The results indicate that the level of the pro-inflammatory IL-1β was greatest at the lowest APS concentration (100 μg ml^{-1}) and was thereafter negatively correlated with the dose. In contrast, both the TGF-β and TNF-α responses were greatest in the center of the APS dose curve at 400 μg ml^{-1}; at all other concentrations, the levels were below those of the non-stimulated control cells. The amount of anti-inflammatory IL-10 secreted by macrophages was greatest at an APS concentration of 100 μg ml^{-1} (Fig. 6A–D). To explore the effect of APS on macrophages after LPS stimulation, macrophages were stimulated with 2 μg ml^{-1} LPS, followed by different concentrations of APS. Cells stimulated with LPS alone were used as a positive control group. After addition of APS to macrophages stimulated by LPS, the amount of TNF-α and IL-1β released was significantly reduced and this was dependent on the concentration of APS. This was especially apparent at 800 μg ml^{-1}, where TNF-α and IL-1β were at minimal levels, indicating that APS can inhibit LPS stimulation of macrophages. In contrast, there was no significant pattern in the expression of TGF-β and IL-10 (Fig. 6E–H).

Cytokines that are secreted during acute phase responses are rapidly synthesized in macrophages and gene regulation is primarily transcriptional but also relies on mRNA stability. Therefore, we measured the effect of APS on steady-state mRNA levels for all 4 cytokines as well as key TLRs since these are also essential to primary immune responsiveness in fish. The mRNA levels for TNF-α, TGF-β, IL-1β, and IL-10 were all elevated in response to APS addition, but only the relative expression of TNF-α mRNA was obviously more than that in the control group at an APS concentration of 800 μg ml^{-1}, showing a strongly significant difference ($p < 0.01$) (Fig. 7A). Expression of the Toll-like receptors TLR2, TLR3, TLR5a, and TLR5b did not change significantly after addition of 600 μg ml^{-1} APS. In contrast, following

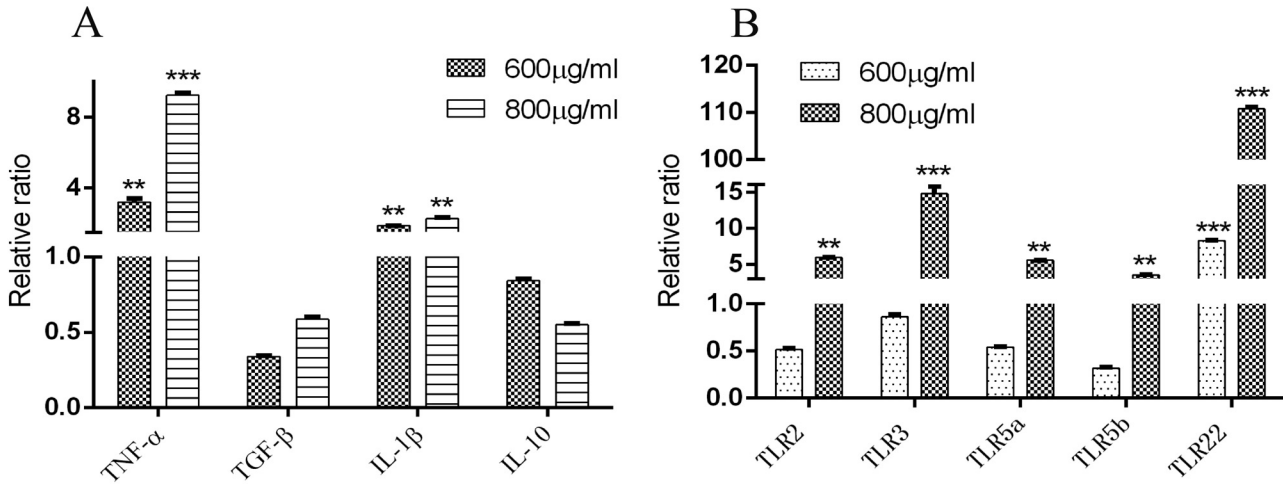

Fig. 7. Expression of (A) the cytokines TNF-α, TGF-β, IL-1β, and IL-10, and (B) the receptors TLR2, TLR3, TLR5a, TLR5b, and TLR22 following stimulation of turbot head kidney macrophages with 600 and 800 μg ml^{-1} APS. **$p < 0.05$; ***$p < 0.01$

the addition of 800 μg ml^{-1} APS, the relative expression of TLR22 mRNA was clearly greater than that in control group, showing a strongly significant difference ($p < 0.01$) (Fig. 7B). This indicates that TLR22 is most likely the primary binding receptor used by these macrophages in response to APS.

4. DISCUSSION

In this study, we investigated the effects of polysaccharides on the head kidney macrophages of turbot *Scophthalmus maximus L.*, aiming to explore their immunomodulatory potential. Through a comprehensive analysis, we observed significant immune responses induced by APS treatment, shedding light on its mechanisms of action and potential applications in aquaculture.

Our isolation and culture of turbot macrophages yielded cells with typical morphological characteristics, consistent with macrophages from other fish species (Secombes et al. 1990). These cells exhibited active proliferation and maintained their structural integrity over extended culture periods, indicating their suitability for immunological studies.

Notably, our findings revealed that APS treatment elicited dose-dependent responses in macrophage function. At an optimal concentration of 800 μg ml^{-1}, APS significantly promoted macrophage proliferation while regulating the production of NO and ROS. These results suggest a dual role for APS in promoting immune cell growth while modulating key effector molecules involved in immune responses.

Proteomic analysis further elucidated the molecular mechanisms underlying APS-mediated immunomodulation. APS treatment induced significant alterations in the expression of immune-related proteins, particularly those involved in NF-κB signaling and cytokine receptor pathways. This suggests that APS exerts its immunostimulatory effects by modulating key signaling pathways associated with immune regulation.

Furthermore, our study identified TLR22 as a potential receptor for APS in turbot macrophages. Toll-like receptors and cytokines are key elements of cellular immunity (Harashima et al. 2012, Bhaskar et al. 2016, H. Zhang et al. 2020, Wang et al. 2021, J. Zhang et al. 2021). This finding is particularly significant as TLR22 is unique to aquatic animals (Aoki et al. 2008, Efferth & Oesch 2021) and plays a crucial role in recognizing pathogen-associated molecular patterns (Fierro-Castro et al. 2012, Samanta et al. 2012, Reyes-Becerril et al. 2015, Liu et al. 2017, Wang et al. 2018, Kumar 2019, Stenberg et al. 2019, Zhan et al. 2019, Sahoo 2020, Naya-Català et al. 2021). The upregulation of TLR22 mRNA expression in response to APS treatment implicates its involvement in mediating the immunomodulatory effects of APS on turbot macrophages.

Moreover, our results demonstrate the anti-inflammatory properties of APS, as evidenced by its ability to attenuate LPS-induced pro-inflammatory cytokine secretion in macrophages. This suggests that APS may have therapeutic potential in mitigating inflammatory responses associated with bacterial infections in aquaculture settings.

Overall, our study provides valuable insights into the immunomodulatory mechanisms of polysaccharides, particularly APS, in turbot macrophages. By elucidating the molecular pathways involved, we lay

the groundwork for future research aimed at harnessing the therapeutic potential of polysaccharides for enhancing disease resistance and promoting growth in aquaculture species like turbot.

5. CONCLUSIONS

To conclude, the present study suggests a dual role for APS in promoting immune cell growth while modulating key effector molecules involved in immune responses. Moreover, our results provide evidence for anti-inflammatory properties of APS. As such, APS could be a candidate immunopotentiating agent for fish. Finally, our findings advance the knowledge of using APS for the treatment of inflammatory diseases in fish.

Data availability. All data generated or analyzed during this study are included in this published article.

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