frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 14 December 2022
DOI 10.3389/fmars.2022.1041681

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

RECEIVED 11 September 2022
ACCEPTED 28 November 2022
PUBLISHED 14 December 2022

CITATION
Yan S, Lyu L, Wang X, Wen H, Li Y, Li J, Yao Y, Zuo C, Xie S, Wang Z and Qi X (2022) The anti-inflammatory cytokine IL-22 exhibited a proinflammatory effect in the ovary aiding with the parturition of ovoviviparous black rockfish (*Sebastes schlegelii*). *Front. Mar. Sci.* 9:1041681. doi: 10.3389/fmars.2022.1041681

The anti-inflammatory cytokine IL-22 exhibited a proinflammatory effect in the ovary aiding with the parturition of ovoviviparous black rockfish (*Sebastes schlegelii*)

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Introduction: As a unique type of immunological process, pregnancy and subsequent parturition are associated with a series of inflammatory events. As an anti-inflammatory cytokine, IL-22 has been proven to participate in the parturition process in mammals. However, less is known about ovoviviparous teleosts. To investigate the role of IL-22 in fish parturition, black rockfish, an ovoviviparous teleost, was used. This fish is unique in that it provides over 60% nutrition supply for over 50 thousand embryos at the same time though a placenta-like structure during pregnancy while delivering all fries in two hours.

Methods: Sequence alignment, phylogenetic tree analysis and homology modeling were performed on IL-22 and its receptor. *In situ* hybridization demonstrated ovarian localization of *il22* and *il22r1*. The protein of rIL-22 was obtained through the prokaryotic expression. RNA-seq analysis was performed on black rockfish ovarian cells treated with rIL-22.

Results: *In situ* hybridization results showed that both *il22* and *il22ra1* were localized in the perinatal follicle layer and embryonic envelope. The *il22* expression level was significantly increased during parturition compared to before and after parturition ($P<0.05$). To further understand the mechanism of IL-22 in parturition, rIL-22 was obtained. The downstream variation genes in primary cultured ovarian cells in the perinatal period were analyzed according to the transcriptomic results. A total of 168 differentially expressed genes (DEGs) were identified in the rIL-22 group compared with the PBS control group (padj < 0.05 and |log2FoldChange| 1). Of those, 134 DEGs were identified in the LPS and

rIL-22 treatment groups, including chemokine, cytokine, and PG synthesis. 34 DEGs were mainly identified in immune-related pathways and reorganization of the cytoskeleton responded only to rIL-22 stimulation but not LPS. KEGG pathways mainly included immune response, PG synthesis, cell death and angiogenesis (p value < 0.05).

Discussion: Taken together, our results indicated that IL-22 plays an important role in the parturition of black rockfish by upregulating PG synthesis and increasing chemokine, proinflammatory factor, as well as PG synthetase levels.

KEYWORDS

interleukin-22, parturition, black rockfish, RNA-seq, cytokine

1 Introduction

Teleost, the most abundant vertebrate, exhibits various reproductive strategies, including viviparity, oviparity and ovoviviparity. As the transitional strategy between viviparity and oviparity, ovoviviparity presents a lecithotrophic source homologous to egg-laying oviparous teleosts. And the internal fertilization in ovoviviparous species is similar to viviparous species. Furthermore, ovoviviparous teleosts also exhibit parturition behavior similar to mammals (Venkatesh et al., 1992; Lyu et al., 2021; Lyu et al., 2022). Parturition is the period and process when the fetus separates from its mother and becomes an independent individual. Parturition is a crucial reproductive event for the continuation of species. As an intriguing immunological event, the onset of parturition expresses a series of immunomodulatory effects during pregnancy. Research in the field of immunology of pregnancy has investigated the possibility that cellular immune effectors participate in these pregnancy complications. In particular, the effects that cytokines have on the fetus and thus on the success and failure of pregnancy have been investigated as the triggers of inflammation (Mazaki-Tovi et al., 2007; Raghupathy and Kalinka, 2008). In human parturition, the levels of proinflammatory cytokines were significantly increased without any infection in the amniotic fluid, uterine tissue, membranes, or maternal serum (Hadley et al., 2018). This indicated that these proinflammatory factors have a vital function in the initiation of parturition, in addition to inducing immunological responses. During pregnancy, the mother undergoes a state of immunosuppression to protect the fetus from maternal immune rejection (Abu-Raya et al., 2020). At the end of pregnancy, the changes to parturition in mammals are accompanied by the transition of the endometrium and the amniotic membrane from a static state to a contractile state. One leading reason is that the uterine environment gradually changes to a proinflammatory environment (Conde-Agudelo and Romero, 2014).

Interleukins (ILs) are regarded as local inflammatory mediators in gestational tissues during pregnancy and parturition. Studies have shown that IL-1 is an essential mediator in embryo implantation and the establishment of pregnancy in mammals (Paulesu et al., 2005). It was also revealed that IL-6 was crucial for controlling the progression of parturition by regulating genes involved in the prostaglandin (PG)-mediated uterine activation cascade (Robertson et al., 2010). In addition, ILs have been shown to play a role in the regulation of ovarian functions in teleosts. It is well established that ovulation is similar to inflammatory responses (Liu et al., 2017; Chatterjee et al., 2020). Treatment with IL-6 could induce a marked increase in the ovulation rate by activating matrix metalloproteinase (MMP) and PG synthesis in climbing perch (*Anabas testudineus*) (Chatterjee et al., 2020). Studies on orange-spotted grouper (*Epinephelus coioides*) and sea bass (*Dicentrarchus labrax L.)* have shown that recombinant IL-1β (r IL-1β) can also promote the expression of prostaglandin G/H synthase 2 (*ptgs2*), a key enzyme in PG synthesis (Buonocore et al., 2005; Lu et al., 2008).

IL-22 is a novel cytokine in the IL-10 family produced by T cells and natural killer (NK) cells (Dudakov et al., 2015). IL-22 exhibited both proinflammatory and anti-inflammatory responses. The double responses depend on the tissue microenvironment including the cytokine environment (Alabbas et al., 2018). IL-22 has been observed in both mice and humans to influence the production of other proinflammatory cytokines, including IL-6, IL-8 and tumor necrosis factor alpha (TNF-α), to coordinate the inflammatory response (Andoh et al., 2005; Kong et al., 2012). In addition, IL-22 plays a dual role in pregnancy and parturition. It has been shown that the increased expression of *il22* in unexplained recurrent pregnancy loss (uRPL) patients may be the cause of endometrial homeostasis disorder and recurrent miscarriage

(Heidari et al., 2021). In endometriosis, the expression of *il22* is significantly upregulated and induces C-C motif chemokine 2 (CCL2) production to recruit macrophages (Mei et al., 2019). More recently, IL-22 was found to cause fetal injury in the amniotic cavity leading to neonatal death. However, it may also participate in host defense against microbial invasion of the amniotic cavity (Gershater et al., 2022). In mice, IL-22 acts as an anti-inflammatory cytokine and prevents premature parturition by inhibiting placental cell death (Dambaeva et al., 2018). In the human uterus, IL-22 secreted by decidual stromal cells (DSCs) and decidual natural killer (dNK) cells promotes the survival of trophoblasts and maintains pregnancy by combining with the receptor (Wang et al., 2013). These studies suggest that IL-22 has the dual role of promoting miscarriage and maintaining pregnancy. Although there have been some studies of IL-22 in mammalian pregnancy and parturition, most of them focus on the molecular level. Therefore, research on nonmammals and the addition of bioinformatics can provide a broader basis for the role of IL-22 in parturition.

Black rockfish (*Sebastes schlegelii*) is an important commercial aquaculture species and performs an ovoviviparous reproductive strategy (Zhang et al., 2020). Females mate with males and store sperm inside the ovary from December until April of the following year when the oocytes mature and fertilization is complete (Wang et al., 2021). The female then gives birth after approximately one month of gestation (Liu et al., 2019). During late gestation and the final parturition period, female individuals always suffer from inflammatory symptoms in the ovary and cloaca, including hyperemia and swelling (Lyu et al., 2022). In the present study, to clarify the possible role of IL-22 in the parturition of black rockfish. We first investigated the tissue expression pattern and ovarian localization of IL-22 in black rockfish. We also analyzed the rna-seq obtained from ovarian cells treated with recombinant IL-22 (rIL-22). Our findings demonstrated the function of IL-22 in ovoviviparous teleosts for the first time and provided evidence for the conserved function of cytokines in the reproductive system during evolution.

2 Materials and methods

2.1 Fish and sampling

All animal experiments in this research were approved by the Animal Research and Ethics Committees of Ocean University of China prior to the initiation of the study. No endangered or protected species were involved in this experiment. All experiments were performed in accordance with the relevant guidelines and regulations.

Experimental individuals were anesthetized with 100 ng/mL 3-aminobenzoate methanesulfonic acid (MS-222) to reduce pain before sacrifice and treatment. The fish used in the experiment were from the aquaculture population maintained in marine cages offshore of Rushan, Shandong, China (36.92°N, 121.54°E). A total of 26 female black rockfish were selected randomly for the experiment (over 3 years old, body weight: 1.0 ± 0.4 kg, body length: 24.3 ± 1.3 cm). All the animals were housed for 2 days in indoor cement pools with a culvert system before the experiment. Whole tissue samples, including heart, liver, spleen, kidney, head kidney, intestine, gill, skin, pituitary, muscle, brain and ovary samples, were collected from three females in December 2021. In April 2022, spleen and ovarian stroma cell cultures were performed on gestational female individuals, and parts of the ovary samples were fixed in 4% paraformaldehyde for *in situ* hybridization. The ovarian tissues were sampled at time points of 24 hours before delivery (n=5), during delivery (n=3), and 24 hours after delivery (n=4). For the "before parturition" samples, the swollen cloaca of maternal fish was full of squeezed fries (Lyu et al., 2022). Since a large number of fries (approximately 50,000 fries) will be delivered, the parturition process normally takes approximately one hour in which we collected the "during parturition" sample. For the "after parturition" samples, we recorded the parturition start time of each maternal fish and collected ovary samples 24 hours later.

2.2 Total RNA extraction and reverse transcription

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, America) according to the manufacturer's instructions. RNA quantity and purity were assessed by a Biodropsis BD-1000 nucleic acid analyzer (OSTC, China) and electrophoresis using a 1% agarose gel. cDNA was prepared using the Prime ScriptTM RT Real Time Kit with gDNA Eraser (Perfect Real Time) (TAKARA, Japan) according to the manufacturer's instructions.

2.3 Quantitative real-time PCR

The ChamQTM SYBR Color qPCR Master Mix (High Rox Premixed) kit (Vazyme, China) was used for qPCR according to the reagent instructions. The qPCR procedure was 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The threshold circulation (C_T) values of each sample were measured using *18S* rRNA (Accession number: KF430619.1) as an internal reference gene (Liman et al., 2013). Three replications of qPCR were performed to confirm the results using the $2^{-\Delta\Delta CT}$ method. The primers for qPCR are shown in Table 1.

2.4 Molecular characterization of *il22* and its receptor cDNAs

The cDNA sequences of the black rockfish *il22*, *il22* receptor alpha 1 (*il22ra1*) and *il22* receptor alpha 2 (*il22ra2* or *il22 bp*)

TABLE 1 primers sequences used for ORF cloning, ISH and qPCR.

Primers	Sequence (5'-3')
Primers for ORFs clone	
il22-orf-F	ACAGAGACATCTTCACAACAGC
il22-orf-R	TGTTCAGTAGAAACAGTCGAGG
il22ra1-orf-F	GCCCACTTACCTAATGAAGATGTG
il22ra1-orf-R	AGCCACACAGCAGATAGC
il22ra2-orf-F	GTCATGACTCGTCTGCTGCTC
il22ra2-orf-R	GCGCTACTCTTGAGGGGG
Primers for pET-C-His vector construction	
pET-il22-F	GTAAAATCGAAGAAGGTCTCCCTGTCGACCGCTCAC
pET-il22-R	GTTACCAGAGGTACCGTTTGCGTCGGCAGCGG
Primers for ISH probe preparation	
il22-orf-F	CGCATTTAGGTGACACTATAGAAGCGTGATCCTGATTGGCTGGATCG
il22-orf-R	CCGTAATACGACTCACTATAGGGAGACATCGTGCAGGTAAGTGAACAGG
il22ra1-orf-F	CGCATTTAGGTGACACTATAGAAGCGTAACGTCCGTTGTGTTCGTG
il22ra1-orf-R	CCGTAATACGACTCACTATAGGGAGACATGACACTGCGTCATCTGTGC
Primers for qPCR	
il22 -F	ACGCCAACATCCTCGACTAC
il22 -R	TGGTGATGGTCGTGATAGCG
il22ra1-F	ACGCAGTGTCATCCCTCAAC
il22ra1-R	TGTTGCAGTAGGGCTGTGTT
il22ra2-F	CCAAAATCAGCTCTCCTGTGTTC
il22ra2-R	CCGGTGTGCATGAGGTAGATG
il6-F	GCTCTGTTGCTGTGTGCTC
il6-R	CCACACCTCCTCCTCACCT
acod1-F	GTCCCCTACACAGAGCAGTC
acod1-R	CCTTTGCGAGCAGCATTTCC
endod1-F	TGCAGTTGGAAGGAACGACC
endod1-R	ATGCGCCTCACATCATAGCC
il12b-F	CCAGACGGGGAATACCTCAAC
il12b-R	TTCCTCCAGTATGACGGTCC
bcl2-F	TGCTGGAGAATGACGGATGG
bcl2-R	CAGTATCGCCTGTGAGGGAC
ppp1r9b-F	TGACATCCACATCTCGGTGG
ppp1r9b-R	CGACTGTTCGTCTCTGAGCA
irrn1-F	AGCCTCAACAGTGAAGGGAC
irrn1-R	AGTGCCGTTCACCCGAATAG

genes were cloned and obtained from black rockfish genomic data (PRJNA516036). Primers for cloning *il22* and *il22r* were designed using Primer5 software (Premier, Canada). The primers for the full-length cDNA sequences of *il22* and *il22r* are shown in Table 1. The 2×Phanta Max Master Mix (Dye Plus) (Vazyme, China) was used for cloning, and the PCR program used was 95°C for 3 min, 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 2 min, followed by 72°C for 5 min, with a final hold at 4°C. The PCR product was purified and cloned into the pCE2 TA/Blunt-Zero vector (Vazyme, China) for subsequent sequencing. The cleavage sites of the signal peptides were predicted using the SignalP 6.0 program (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight and isoelectric point were predicted using the ExPASy Compute pI/MW tool (http://web.expasy.org/compute_pi/). Multiple sequence alignments were generated by the ClustalX 2.1 and Espript 3.0 programs (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The phylogenetic tree was reconstructed from the multiple alignments of the amino acid sequences with the neighbor-joining method using MEGA11 (Tamura et al., 2021). The values on the trees represent bootstrap scores of 1,000 iterations, indicating the credibility of each branch. The 3D structures of black rockfish IL-22/IL-22R1/IL-22R2 were modeled using SWISS-Model (https://swissmodel.expasy.org/).

2.5 Recombinant expression of IL-22

rIL-22 was expressed by the *Escherichia coli* Rosetta-gami B (DE3)/pET series vector (Novagen, Germany) system and purified by a Ni-NTA column (Beyotime, China). The primers with overlaps for IL-22 were designed to amplify the sequence encoding IL-22 (Table 1). After digestion with *Bam*H I and *Kpn* I, the amplicon was cloned into the pET-C-His expression vector, and the constructed plasmid was subsequently transformed into *E. coli* Rosetta-gami B (DE3). See Supplementary 1 for details.

The positive clone of *E. coli* Rosetta-gami B (DE3) was grown in Luria Broth (LB) medium with glucose (2 g/L, Sigma–Aldrich), ampicillin (100 mg/L, Sigma–Aldrich, St. Louis, MO, USA) and chloramphenicol (34 mg/L, Sangon Biotech, Shanghai, China) in a shock incubator at 37°C for 4 h. When the OD_{600} ranged from 0.4 to 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma –Aldrich) was added at a final concentration of 0.4 mM, and the temperature was lowered to 16 °C. After 14 h of induction, the cells were collected by centrifugation at 3,500 × g for 10 min at 4° C. The cells were resuspended in lysis buffer (500 mM NaCl, 20 mM phosphate buffer, 10 mM imidazole, and pH 7.4). The sample was ultrasonically decomposed on ice, followed by centrifugation at 12,000 g for 10 min at 4°C to retain the supernatant. The supernatant was collected and filtered through a 0.22 μm filter. The rIL-22 in the supernatant was purified on a Ni-NTA column. The concentration of rIL-22 was determined by a BCA kit (Beyotime Biotechnology, Shanghai, China), and the purified protein was analyzed by SDS–PAGE. The purified rIL-22 was frozen in liquid nitrogen and stored at -80°C.

2.6 *In situ* hybridization

Following the manufacturer's instructions, the 2 × Phanta® Max Master Mix (Dye Plus) kit (Vazyme, China) was used for PCR, and the cDNA of the ovary was used as a template. Following the manufacturer's instructions, the DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland) was used to synthesize antisense or sense probes for the *in vitro* transcription of *il22* and *il22ra1* mRNA. The specific process of ISH was performed as previously reported (Wang et al., 2022).

2.7 Culture of ovarian cells

We obtained ovarian cells by removing the ovarian parietal membrane, embryo, fries and large oocyte with forceps and scissors. The cells were cultured for 48 h in L15 (G-Clone, China, Beijing) complete medium with 10% fetal bovine serum (FBS) (G-Clone, China, Beijing) and 1% penicillin–streptomycin-gentamicin solution (Absin, China, Shanghai). Subsequently, L15 starvation medium without FBS but containing 1% penicillin–streptomycin-gentamicin solution was used to culture the cells for 12 h under low-transcription conditions. Finally, complete medium with PBS (solvent control, n=3), lipopolysaccharides (LPS, final concentration: 100 ng/mL, n=3), and rIL-22 (final concentration: 500 ng/mL, n=3) was used to treat cells for 6 h. The LPS group was used as a positive control to induce inflammation in ovarian cells. Subsequently, the cells were harvested for RNA extraction.

2.8 RNA isolation and library construction

RNA isolation of each cell sample was performed as shown previously. Qualities and concentrations of total RNA were evaluated by a NanoDrop (Thermo Fisher Scientific, USA) and an Agilent 2100 bioanalyzer system (Agilent Technologies, USA). The RIN (RNA Integrity Number) value of each sample was above 9. The NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) was employed to generate 9 sequencing libraries according to the manufacturer's instructions. The samples were sequenced on an Illumina HiSeq X Ten platform, and 150-bp paired-end reads were generated.

The transcriptomic data obtained by removing the reads of adaptors were aligned to the reference *Sebastes schlegelii* genome (PRJNA516036) with HISAT2 (Kim et al., 2015a). Assemble and quantification analyses were accomplished with the StringTie package (Pertea et al., 2016). The multiple mapped reads were removed, and the count numbers of unique mapped reads and FPKM (Fragments Per Kilobase Per Million) were retrieved and normalized with previous references (Anders et al., 2015). Principal component analysis (PCA) was performed by the ggplot2 package.

Based on the DESeq2 package, statistical analysis of transcripts with a cutoff "padj" < 0.05 and absolute fold change values greater than 1 were marked as significantly differentially expressed genes (DEGs). DEG annotation was based on the reference *Sebastes schlegelii* genome. Thereafter, DEGs were assigned to Gene Ontology (GO) classification by the aid of the Blast2GO program with the p value threshold < 0.05 (Götz et al., 2008). Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis (KEGG, a database of biological systems, http://www.genome.jp/kegg/) was performed on significant pathway enrichment analysis. The clusterProfiler R package was employed to test the statistical enrichment of DEGs in KEGG pathways with the p value threshold < 0.05 (Kanehisa et al., 2017). The STRING database was used to construct a protein–protein interaction (PPI) network with *Homo sapiens* used as a reference species.

2.9 Statistical analysis

All the data are shown as the mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by Tukey's and Sidak's multiple range tests. Differences were considered significant at $P < 0.05$. All statistical procedures

and graphs were generated with GraphPad Prism 9 (GraphPad Software, USA).

3 Results

3.1 Molecular cloning and characterization of *il22* and its receptors

The cDNA sequences of *il22* (Accession number: OP331275), *il22ra1* (Accession number: OP331276) and *il22ra2* (Accession number: OP331277) were identified and cloned based on black rockfish genomic data. The ORFs of *il22*, *il22ra1* and *il22ra2* were 567 bp, 1671 bp and 612 bp, encoding 188 amino acids, 556 amino acids and 203 amino acids, respectively. The physicochemical properties of the IL-22 protein are as follows: the molecular weight is 18.17 kDa, and the theoretical isoelectric point is 6.62. According to amino acid sequence analysis, IL-22 from black rockfish has six α-helices and two adjacent disulfide bridges. Meanwhile, the amino acid homology of IL-22 in black rockfish is high compared with that in Nile tilapia (*Oreochromis niloticus*) (Figure 1A). However, the amino acid sequences of IL-22RA1 and IL-22RA2 were not conserved (Figure 1B). The phylogenetic analysis showed that IL-22 and its receptors were clustered with teleost and differed from other vertebrates (Figures 1C, D).

3.2 Expression patterns and localization of il22 and its receptors

Tissue distribution analysis showed that *il22* and its receptors were widely expressed in various tissues. In particular, *il22* was highly expressed in the intestines, gills and skin of black rockfish. In addition, a certain amount of *il22* was detected in the ovary (Figure 2A1). The tissue expression patterns of *il22ra1* and *il22ra2* were similar to *il22*. High *il22ra1* mRNA levels were observed in the intestines, gills, skin, and ovary (Figure 2A2). The *il22ra2* transcript is widely expressed in different tissues. Compared with *il22ra1*, *il22ra2* was highly expressed in the kidney and head kidney (Figure 2A3). The *il22* expression pattern in the black rockfish perinatal period showed that *il22* was significantly increased during parturition ($P<0.05$) (Figure 2B).

The embryo development and mastery exchange in black rockfish was determined by the maternal-fetal interface. The maternal-fetal interface consists of the follicle layer, embryonic

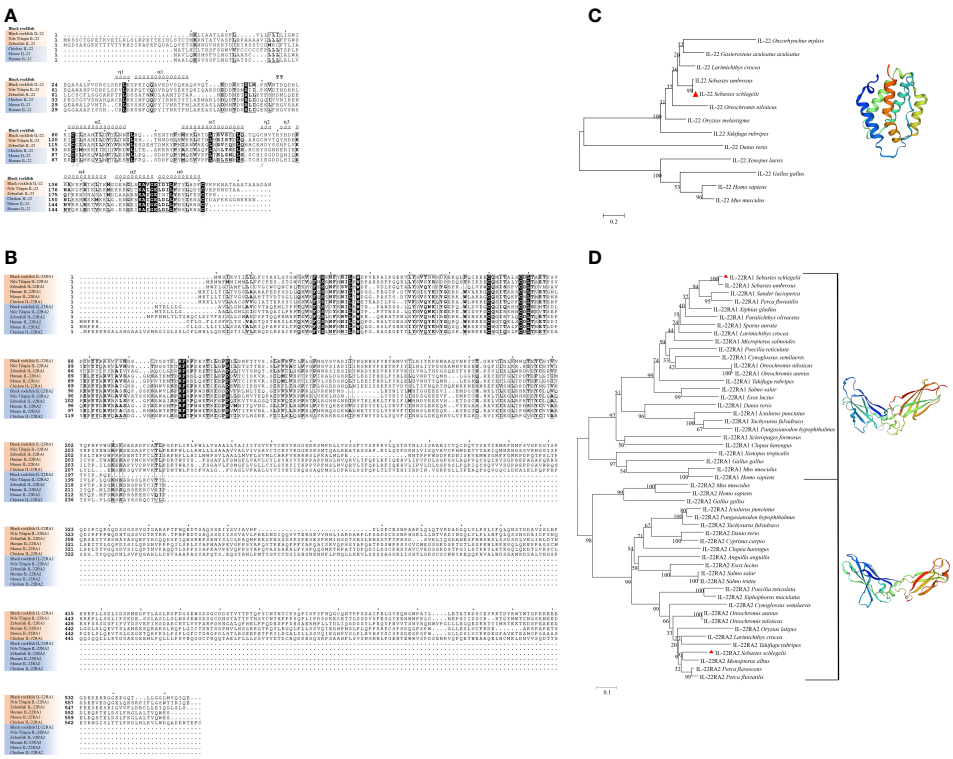

FIGURE 1
Multiple sequence alignment of IL-22 **(A)**, IL-22RA1 and IL-22RA2 **(B)** in black rockfish, nile tilapia, zebrafish, chicken, mouse, and human. Phylogenetic tree of IL-22 **(C)**, IL-22RA1 and IL-22RA2 **(D)** in black rockfish and other species. Data were resampled with 1,000 bootstrap replicates. The access number of the sequences used to generate the multiple sequence alignment and phylogenetic tree were listed in Supplementary 2.

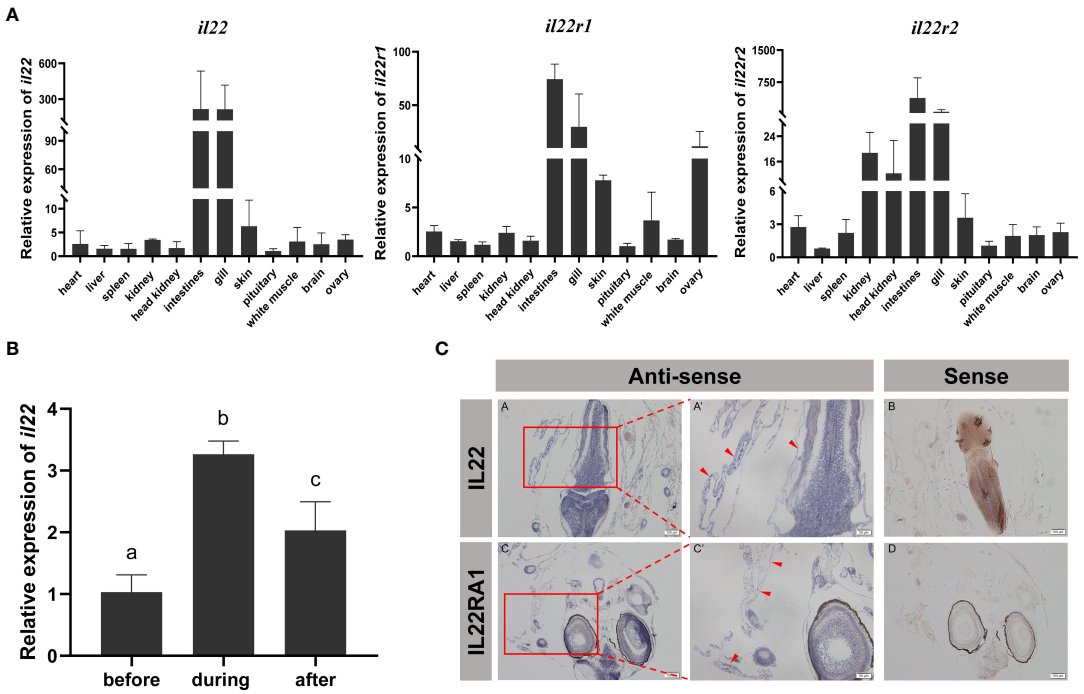

FIGURE 2
Relative expression levels of *il22* **(A1)**, *il22ra1* **(A2)** and *il22ra2* **(A3)** in different organs (heart, liver, spleen, kidney, head kidney, intestine, gill, skin, pituitary, white muscle, brain, and ovary) (n = 3). The X axis indicates different tissues. The Y axis indicates the relative expression normalized to *18s* rRNA. **(B)** Relative expression levels of *il22* in black rockfish before parturition (n = 5), during parturition (n = 3), and 24 h after parturition (n = 4). The X axis indicates different parturition stage of black rockfish. The Y axis indicates the relative expression normalized to *18s* rRNA. The data are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$). **(C)** Localization of *il22* and *il22ra1* mRNA in ovary of black rockfish *via in situ* hybridization. The red arrows indicate positive signals. Scale bars (A–D) = 100 μm. Scale bars (A′, C′) = 50 μm.

envelope and the liquid between them. We performed *in situ* hybridization of *il22* and *il22ra1* on ovaries during the perinatal period. The results showed that *il22* and *il22ra1* positive signals were observed in the follicle layer and embryonic envelope. In embryos, extensive positive signals of *il22* and *il22ra1* were also detected in various tissues, especially in gill, intestine and skin tissues (Figure 2C).

3.3 Prokaryotic expression, purification and functional verification of rIL-22

To further study the molecular function of IL-22, rIL-22 was generated through a prokaryotic expression system. We obtained rIL-22 with a c-terminal 6× His tag soluble in the supernatant after IPTG-induced expression. A single band of approximately 18 kDa was observed on the SDS–PAGE gel after purification by the Ni-NTA column (Figure 3A). The protein concentration of rIL-22 was 0.25 mg/mL, as determined by a BCA kit. Primary cells from black rockfish spleen were cultured to verify the bioactivity of rIL-22. As shown in Figure 3C, the rIL-22-treated group did not differ from the control group, but rIL-22 significantly reduced LPS-induced *il6* and *ptgs2* levels when combined with LPS ($P<0.05$). These results indicated that rIL-22 was functionally active *in vitro*.

To understand the role of IL-22 in ovarian cells during the perinatal period, we treated ovarian cells with different concentrations of rIL-22 with or without LPS. In contrast to the results in the spleen, the *il6* level in ovarian cells was increased along with the rIL-22 concentration with or without LPS Figure 3B).

3.4 RNA-seq revealed the function of IL-22 in black rockfish ovaries

To verify the response mechanism of IL-22 in black rockfish ovaries during the perinatal period, nine groups of cDNA libraries were constructed and sequenced on the Illumina platform (Accession number: PRJNA876649). By high-throughput sequencing, 44,521,866, 46,205,972 and 48,020,019 average raw reads were obtained from the PBS, LPS, and IL-22 groups, respectively. After filtration, the average clean reads of the control and treatment groups used in subsequent analysis were 43,498,905, 45,496,249 and 47,222,700, with Q30 average percentages of 92.67, 91.93 and 91.76%, respectively. By mapping these clean reads with black rockfish genomes, the

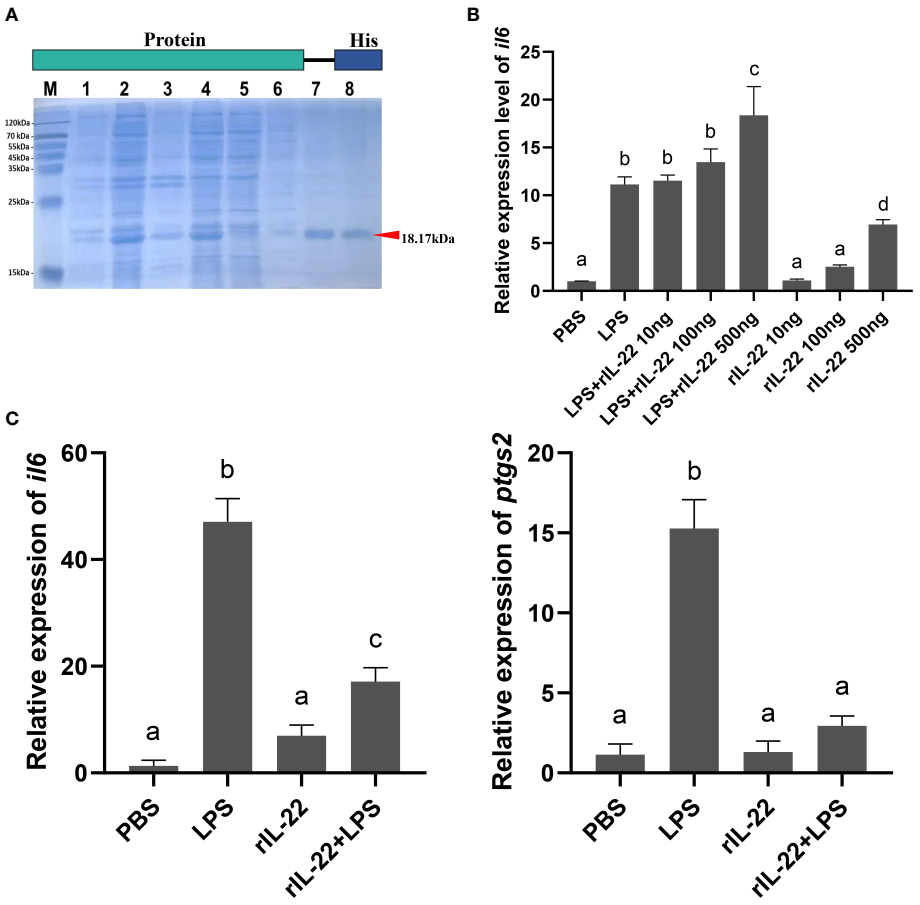

FIGURE 3
(A) SDS-PAGE analysis of purified rIL-22 (M: Marker; lane 1: Total protein in the thallus before induction; lane 2: Total protein in thallus after induction; lane 3: Protein in the precipitate after induction; lane 4: Protein in the supernatant after induction; lane 5, 6: The non-target proteins are washed down; lane 7, 8: Purified rIL-22 (18.17 kDa)). **(B)** Relative expression of *il6* under PBS and different concentrate LPS and rIL-22 stimulation (n=3). **(C)** Relative expression of *il6* and *ptgs2* after PBS, LPS, rIL-22 and rIL-22 + LPS incubation, respectively (n=3). The X axis indicates different treatments. The Y axis indicates the relative expression normalized to *18s* rRNA. All data are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

average total map of the PBS, LPS, and IL-22 groups was obtained as 40,352,149.33 (92.74%), 42,316,499 (93.01%), and 43,825,848 (92.80%), respectively.

The PCA showed significant differences among the PBS, LPS, and IL-22 groups (Figure 4A). We identified 760 significant DEGs in the LPS group compared to the PBS group, including 528 upregulated DEGs and 232 downregulated DEGs. Simultaneously, 168 significant DEGs were identified in the IL-22 group compared with the PBS group, including 143 upregulated DEGs and 25 downregulated DEGs. In addition, 66 significant DEGs were detected in the IL-22 group compared to the LPS group, including 51 upregulated DEGs and 15 downregulated DEGs (Figure 4B–D).

A total of 134 DEGs were clustered in the intersection between the LPS vs. PBS groups and IL-22 vs. PBS groups, which stood for the coactivated genes of both LPS and IL-22 (Figure 5A). The heatmap indicated that the expression patterns of these 134 DEGs were similar in the IL-22 and LPS groups (Figure 5B). Further annotation of these 134 DEGs is presented in Figures 5C, D. KEGG enrichment analysis identified 20 pathways, which were considered to be related to the following: *immune response* (13 pathways), *PG synthesis* (3 pathways), *angiogenesis* (2 pathways) and *cell death* (2 pathways) (Figure 5C). GO enrichment analysis showed that the top 10 GO terms in the Biological Process were classified into four categories: *immune response* (GO: 0006955, 0002376), *fatty acid metabolism* (GO: 0006633, 0006631, 0044255), *stress response* (GO: 0006979, 0006950), and *monocarboxylic acid* (GO: 0072330, 0032787) (Figure 5D). The PPI analysis revealed a complex network of interactions among 134 DEGs in which chemokines, cytokines and PG synthesis-related proteins were the main focus (Figure 6A). In addition, 34

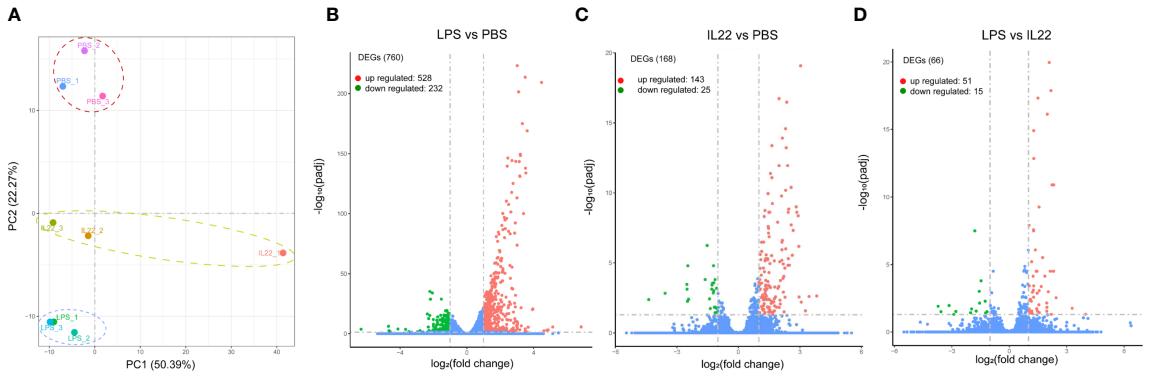

FIGURE 4
(A) PCA of the nine libraries treated with PBS (PBS_1, PBS_2, PBS_3), LPS (LPS_1, LPS_2, LPS_3), and IL-22 (IL22_1, IL22_2, IL22_3), respectively. Volcano plots represented the DEGs in LPS vs. PBS group **(B)**, IL-22 vs. PBS group **(C)**, and LPS vs. IL-22 group **(D)**, respectively.

DEGs were identified and responded only to IL-22 stimulation. There were particular interactions between the 34 DEGs through the PPI analysis (Figure 6B). They are mainly classified as immune-related and cytoskeleton reorganization-related. To verify the expression levels of genes in transcriptome data, 7 DEGs were randomly selected for qPCR. As shown in Figure 6C, the gene expression levels obtained by qPCR were basically consistent with the transcriptomic data.

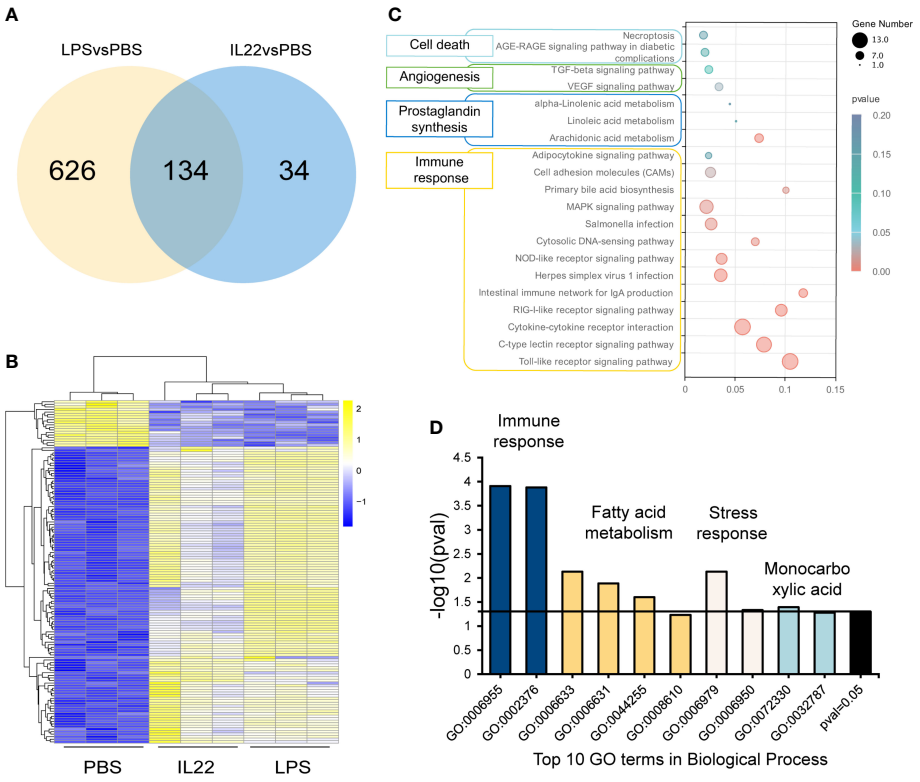

FIGURE 5
(A) Venn diagram of the DEGs of LPS vs PBS group and IL-22 vs PBS group. **(B)** Heatmap of 134 DEGs in the three groups (PBS, LPS, and IL-22). Yellow and blue colors indicated up- and down-regulated transcripts, respectively. **(C)** KEGG analysis of the 134 DEGs. Enriched pathways were classified into four classifications including Immune response, PG synthesis, Angiogenesis, and Cell death. **(D)** The top 10 GO terms of the 134 DEGs were classified into Immune response, Fatty acid metabolism, Stress response, and Monocarboxylic acid.

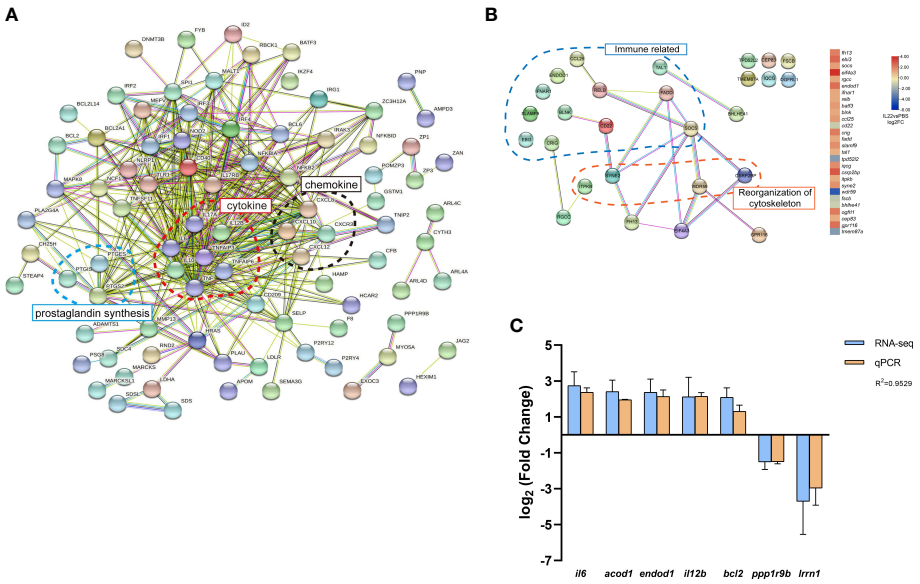

FIGURE 6
(A) The PPI analysis of the 134 DEGs enriched from the intersection of LPS vs. PBS group and IL-22 vs. PBS group. Red circle represented DEGs related to cytokine. Black circle represented DEGs related to chemokine. Blue circle represented DEGs related to prostaglandin synthesis. **(B)** The PPI analysis of the 34 DEGs only expressed in IL-22 vs. PBS group. Red circle represented DEGs related to reorganization of cytoskeleton. Blue circle represented DEGs related to immune related. **(C)** The qPCR validation of seven DEGs generated from the RNA-Seq results. These genes include *il6*, Cis-aconitate decarboxylase (*acod1*), *endod1*, *il12b*, B cell lymphoma 2 (*bcl2*), protein phosphatase 1 regulatory subunit 9B (*ppp1r9b*), and leucine rich repeat neuronal 1 (*lrrn1*).

4 Discussion

IL-22 is a member of the IL-10 cytokine family. Protein modeling revealed that the six α-helices in IL-22 were similar to those in other IL-10 family members (Wolk et al., 2010). The sequence alignment results showed that IL-22 of black rockfish had two adjacent disulfide bridges, which is consistent with a study in teleosts (Siupka et al., 2014). The phylogenetic trees showed that IL-22 congregated with teleosts and differed from mammals. IL-22 acts *via* the IL-22 receptor, which is a heterodimer consisting of IL-22RA1 and IL-10RB (Trevejo-Nunez et al., 2016). The IL-22 receptor complex is a transmembrane complex associated with JAK and TYK. It triggers a cascade of downstream reactions when IL-22 binds to its receptor complex, including phosphorylation of JAK and TYK2 and activation of STAT3 (Wu et al., 2020). In addition, other molecules include P38, ERK, JNK, and PI3K (Sabat et al., 2014). IL-22 also possesses the soluble receptor IL-22RA2, also known as IL-22BP, which can competitively combine with IL-22.

In humans, pregnancy is thought to be a state of immunosuppression, whereas delivery is a reactivation of local inflammation (Osman et al., 2003). Studies have shown that the volume of the uterus occupied by the embryo acts as a signal that is sensed by the uterus (Shynlova et al., 2013a). The plateau reached in embryonic growth may act as a signal to start parturition. In mice, the myometrium cells can sense mechanical stretching signals from the growing embryo (Shynlova et al., 2008). Meanwhile, it has been reported that mechanical stretching can stimulate uterine chemokine expression, such as CCL2 (Shynlova et al., 2008), C-X-C motif chemokine 1 (CXCL1) (Hilscher et al., 2019), CXCL5 (Bollapragada et al., 2009), and CXCL8 (Lei et al., 2011), which aggregate immune cells. The immune cells that accumulate include T cells (Hamilton et al., 2012), neutrophils and macrophages (Gimeno-Molina et al., 2022). These immune cells are recruited from the peripheral circulation to cause decidual infiltration (Shynlova et al., 2013b). The large accumulation of immune cells at the maternal-fetal interface in turn produces extracellular matrix (ECM) degradation proteases such as MMP to promote cervical relaxation (Kelly, 2002), and are also a source of cytokines such as IL-22, IL-1β, IL-6 and TNF-α. IL-22 promotes chemokine and cytokine production. IL-1β could promote prostaglandin E2 (PGE2) synthesis in uterine tissues harvested from pigs (Franczak et al., 2010). IL-6 and TNF-α upregulate the expression of *ptgs2* in different cell types (Honda, 2011; McHale et al., 2018; Koyama et al., 2021). PGE2 is an inducer of parturition due to its increased levels in the uterus during parturition and its ability to cause strong uterine contractions (Sugimoto et al., 2015). During the complex process of parturition, inflammatory cytokines play a very important role.

The *il22* level was significantly increased during the parturition of black rockfish, suggesting that IL-22 plays an important role in parturition. According to the transcriptomic results, 134 DEGs involved in chemokine, cytokine, and PG synthesis were identified in both the IL-22 and LPS groups. A similar expression pattern of these DEGs was also found in the transcriptomic data of the human myometrium (Mittal et al., 2010). These results indicated a conserved mechanism in parturition between mammals and ovoviviparous fish. In our results, IL-22 induced the production of chemokine-related genes such as CXCL10/CXCR3, CXCL12 and CXCL8 in the ovary at parturition. In human preterm parturition, the CXCL10/CXCR3 system can recruit T lymphocytes to cause placental infiltration (Romero et al., 2017). CXCL12 is widely present in placental cells and is considered a key component of fetal outcome (Ao et al., 2020). Higher levels of CXCL8 have been reported to be associated with preterm parturition (Rode et al., 2012). These chemokines recruit immune cells to the maternal-fetal interface to amplify inflammatory signaling cascades.

IL-6 and TNF-α are considered to be involved in parturition through the PG signaling pathway (Keelan et al., 2003). In mice, IL-6 promotes the expression of genes that control PG synthesis and signaling in isolated uterine cells (Robertson et al., 2010). Meanwhile, TNF-α promotes the production of *ptgs2*, which leads to the upregulation of prostaglandin F2a ($PGF2_a$) in bovine endometrial stromal cells (Sakai et al., 2021). In our results, proinflammatory cytokines (IL-6, TNF-α and IL-17) and PG synthesis-related genes (*ptgs2*, *ptgis*, and *ptges*) were upregulated, which is consistent with the results in mammals. The fertilization process of black rockfish occurs in situ, and the parturition process is similar to ovulation in oviparous teleosts. Studies on oviparity ovulation have proven that PGs can activate ovulation in medaka (*Oryzias latipes*) (Fujimori et al., 2011), pacu (*Piaractus mesopotamicus*) (Criscuolo-Urbinati et al., 2012), goldfish (*Carassius auratus*) (Sorensen et al., 2018), catfish (*Heteropneustes fossilis*) (Joy and Singh, 2013), and longchin goby (*Chasmichthys dolichognathus*) (Baek and Lee, 2019). In ovoviviparous guppies (*Poecilia reticulata*), intraperitoneal administration of $PGF2_a$ significantly induces parturition (Lyu et al., 2021). In a previous study on black rockfish, *ptgs2*, a key enzyme in PG synthesis, was also proven to be related to parturition (Lyu et al., 2022). Taken together, IL-22 was involved in parturition by promoting PG synthesis in black rockfish. Meanwhile, rIL-22 significantly upregulated the expression of *ptgs2* and *il6* in the spleen and ovary. The above processes of parturition induced by IL-22 appear to be similar to those induced by pathogenic bacterial infections.

In addition, our transcriptomic data showed that 34 DEGs were mainly enriched in immune-related and cytoskeleton reorganization-related genes that respond to IL-22 stimulation but not LPS. The immune-related genes included fas associated *via* death domain (*fadd*), transcription factor RelB (*relb*), endonuclease domain containing 1 (*endod1*), and suppressor of cytokine signaling 1 (*socs1*), etc. As a ubiquitous adaptor protein, FADD, regulates cell apoptosis and autophagy to maintain homeostasis. Meanwhile, FADD inhibits typical cell death, such as necroptosis, which can trigger inflammatory responses (Mouasni and Tourneur, 2018). Therefore, we speculated that FADD may maintain ovarian homeostasis by regulating cell death. RELB can activate NF-κB through both classical and nonclassical pathways as a key molecule (Madge and May, 2011). Our results showed that RELB was upregulated, suggesting that IL-22 could activate NF-κB through a nonclassical pathway in black rockfish parturition. Studies have shown that RELB can limit acute inflammation and repress innate immunity by blocking or replacing RELA in the classical pathway (Millet et al., 2013). Therefore, we hypothesized that IL-22 may play a limiting role in preventing the expansion of inflammation during the local inflammatory reaction of black rockfish. Our results above showed that inflammation in black rockfish parturition is similar to the inflammation caused by recognition of pathogen-associated molecular patterns (PAMPs). However, ENDOD1 can participate in the degradation of neutrophil extracellular traps (NETs) after pathogen clearance to protect the host from autoimmune damage (Lyu et al., 2016). In addition, SOCS1 is a representative negative regulator of JAK/STAT-mediated cytokine signaling and strictly regulates pathways related to inflammation (Yoshimura et al., 2021). These results suggested that IL-22 may protect the ovary from autoimmune damage and play a limiting role in inflammatory pathways during parturition in black rockfish. The reorganization of cytoskeletal genes, including inositol-trisphosphate 3-kinase B (*itpkb*), WD repeat domain 59 (*wdr59*), and spectrin repeat containing nuclear envelope protein 2 (*syne2*), was also enriched in the IL-22 vs. PBS groups. Studies have shown that the F-actin binding domain of the ITPKB isoenzyme exhibits the ability to reorganize cytoskeletal networks to influence cell movement and migration (Erneux et al., 2016). An important cause of parturition events is ovary contraction due to cytoskeletal remodeling and motility (Taggart and Morgan, 2007). In our results, the *wdr59* gene was significantly downregulated. WDR59 is a component of the GATOR2 protein complex that can indirectly promote mammalian target of rapamycin C1 (mTORC1) activity (Kim et al., 2015b). Activation of mTORC1 can promote cell motility and invasion (Zhou and Huang, 2011). The mechanical connection between the nucleus and the cytoskeleton is provided by nesprins, including *syne2* (also called *nesprin2*). SYNE2 can bind to the cytoskeleton and work with actin to drive cellular nuclei and movement (Davidson et al., 2020). Fertilization and embryo development of black rockfish was processed inside the ovary. It is also indicated that the process of parturition is similar to oviparity ovulation. The process of parturition involves follicular layer and egg envelope rupture, which are all associated with cytoskeletal

remodeling. In our results, DEGs regulated by IL-22 were associated with cytoskeletal remodeling in ovarian cells, suggesting that IL-22 may function in ovarian cell migration or motility during parturition in black rockfish.

IL-22 activates different important pathways in black rockfish parturition. The transcriptomic results included pathways related to the immune response, PG synthesis, angiogenesis and cell death. The activation of the immune response plays an important role during parturition. The Toll-like receptor (TLR) signaling pathway regulates the downstream inflammatory response by activating the transcription factors NF-κB and JNK through the MyD88 adaptor (Takeda and Akira, 2004). It has been shown that TLR4 expression is significantly increased in human pregnancy tissues during preterm and term parturition (Chen et al., 2020). In this study, TLR signaling pathway-related genes were upregulated, similar to research results seen in humans. The TLR signaling pathway may play a role in the activation of inflammatory cytokines in the ovarian stroma during black rockfish parturition. The cytokine–cytokine receptor interaction was also significantly upregulated, which was expected. C-type lectin receptors (CLRs) can trigger a variety of signaling pathways that broadly lead to the activation or inhibition of cellular functions. CLRs can induce a large number of cytokines and chemokines. In general, CLRs induce a proinflammatory response, which can directly or indirectly induce the production of IL-1β, IL-6, TNF and leukotrienes (LTs) (Brown et al., 2018). The CLRs may be involved in the production of cytokines in the parturient period of black rockfish. PG is a key factor in parturition. Correspondingly, PG synthesis was significantly enriched in our results. The results included linoleic acid metabolism and arachidonic acid metabolism. Arachidonic acid can form PGE2 in the presence of *ptgs2* (Wang et al., 2019). However, linoleic acid is an upstream provider that generates arachidonic acid (Szczuko et al., 2020). Research has shown that both full-term spontaneous parturition and preterm parturition lead to a high expression of genes related to angiogenesis (Haddad et al., 2008). Similarly, vascular endothelial growth factor (VEGF) signaling pathway-related genes were significantly upregulated in our results. In human research, VEGF not only plays an important role in decidua growth and maintenance but also may stimulate leukocyte extravasation into the decidua and promote decidua inflammation (Christiaens et al., 2008). The transforming growth factor beta (TGF-β) signaling pathway is a multifunctional cellular pathway. TGF-β in bovine placenta may play an important role in fetal membrane dissection after parturition through cell inhibitory activity and ECM remodeling (Hirayama et al., 2015). Because black rockfish expel their larvae directly at parturient time, TGF-β may play a potential role in the process of the larvae leaving the ovary. Intracellular components released by cell death are one of the stimuli that trigger the proinflammatory gene expression program in innate immune cells (Pasparakis and Vandenabeele, 2015). In our results, cell death-related pathways such as the necroptosis pathway were significantly enriched.

In summary, we propose that IL-22 plays an important role in the parturition of black rockfish by upregulating PG synthesis and promoting parturition-related inflammatory signals. On the one hand, chemokines (CXCL10/CXCR3, CXCL12, and CXCL8) and proinflammatory cytokines (IL-6, TNF-α, and IL-17) caused the destruction of the immune tolerance balance in the ovary and further increased PG synthesis. On the other hand, IL-22 also activated PG synthesis directly by regulating related PG synthetase levels. In addition, IL-22 may play a protective role by maintaining ovarian homeostasis and limiting inflammatory amplification and cytoskeletal remodeling.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject, PRJNA876649.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Ocean University of China.

Author contributions

HW, XQ and YL designed the study. SY performed the experiment. ZW provided all the fish for sampling. SY, LL, XW, JL, YY, CZ and SX performed in samples collection. SY wrote the manuscript, XQ and LL provided manuscript editing and feedback. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by grants from The National Natural Science Foundation of China (41976089).

Acknowledgments

We thank ZW for providing us with all the fish for sampling.

Conflict of interest

Author ZW was employed by the company Weihai Taifeng Seawater Seedling Co.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1041681/full#supplementary-material

SUPPLEMENTARY 1
Details of the prokaryotic expression vector.

SUPPLEMENTARY 2
The GenBank accession numbers and sequences of IL-22, IL-22RA1 and IL-22RA2 screened by the phylogenetic tree.

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