Huan et al. *Zool. Res.* 2025, 46(5): 1137−1152
https://doi.org/10.24272/j.issn.2095-8137.2024.477
https://cstr.cn/32248.14.zr.2024.477

ZR | Zoological Research

Article Open Access

Interleukin-22 functions to alleviate hypoxia-induced intestinal inflammation by modulating pro- and anti-inflammatory factors in *Pelteobagrus fulvidraco*

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ABSTRACT

Intestinal inflammation is a common challenge in intensive aquaculture, yet its pathogenesis remains unclear. While interleukin 22 (IL-22) is recognized as a critical regulator of cellular homeostasis during inflammation in higher vertebrates, its roles in fish are not well understood. This study established hypoxia-induced models in intestinal tissues and primary intestinal epithelial cells of yellow catfish to investigate the involvement of IL-22 in maintaining intestinal homeostasis. Results revealed that *Pelteobagrus fulvidraco* IL-22 (*Pf_IL-22*) was abundantly expressed in mucosal tissues, with the highest levels in the gill and intestine. Hypoxia induced pronounced intestinal injury, characterized by loosening of the lamina propria and extensive vacuolization, while activating hypoxia-inducible factor (HIF) signaling and markedly up-regulating IL-22 expression. IL-22 levels peaked at 24 h post-hypoxia, suggesting a role in early immune responses. Recombinant *Pf*_IL-22 also induced transcription of pro-inflammatory mediators, including IL-1β and tumor necrosis factor α (TNF-α), in primary intestinal epithelial cells, indicating a dual regulatory function in balancing protection and inflammation. Mechanistic analyses revealed that HIF-1α directly interacted with a hypoxia response element within the IL-22 promoter to drive transcription, as confirmed by dual-luciferase assays, electrophoretic mobility-shift assays, and HIF-1α knockdown. Silencing *Pf_IL-22* significantly suppressed Th17 cell differentiation pathways, demonstrating its role in shaping downstream immune responses. These findings establish the HIF-1α/IL-22 axis as a key regulatory pathway modulating immune responses and alleviating intestinal inflammation, providing a basis for developing IL-22-targeted immunotherapies and selective breeding strategies in aquaculture.

Keywords: Interleukin-22; HIF-1α/IL-22 axis; Hypoxia; Intestinal inflammation; Teleost

INTRODUCTION

Intestinal inflammation represents a major pathological challenge in fish cultured under high-density aquaculture conditions, frequently resulting in physiological dysfunction and substantial economic losses. Affected individuals typically exhibit lethargy, loss of appetite, abdominal distention with hemorrhagic exudates, and empty intestines, leading to high mortality rates (Serna-Duque & Esteban, 2020). Cumulative evidence indicates that most fish diseases originate from mucosal injury or infection, particularly within the intestinal epithelium (Cao et al., 2025), which is continuously exposed to diverse microbial, viral, and chemical insults (Rombout et al., 2011; Stosik et al., 2023). Consequently, the intestinal mucosa constitutes a critical barrier and an essential component of fish immunity. Although teleosts lack organized intestinal-associated lymphoid structures analogous to mammalian Peyer patches, the lamina propria (LP) underlying intestinal epithelial cells (IECs) contains abundant leukocytes, including innate immune cells such as macrophages and neutrophils, as well as adaptive immune cells dominated by T lymphocytes (Komatsu et al., 2009; Yu et al., 2020).

Among adaptive immune subsets, Th17 cells play a critical

Received: 29 May 2025; Accepted: 30 July 2025; Online: 31 July 2025

Foundation items: This study was supported by the National Natural Science Foundation of China (32102760), "JBGS" Project of Seed Industry Revitalization in Jiangsu Province (JBGS(2021)034), Creation Project of Major New Species of Agriculture in Jiangsu Province (PZCZ201742), Key Laboratory of Healthy Freshwater Aquaculture, Ministry of Agriculture and Rural Affairs, Key Laboratory of Freshwater Aquaculture Genetic and Breeding of Zhejiang Province; Zhejiang Institute of Freshwater Fisheries, Huzhou 313001 (ZJK202404)

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role in allergic and chronic inflammation within the intestine (Park et al., 2005). These cells secrete a repertoire of cytokines with diverse functions, contributing to the regulation of epithelial barrier integrity and maintenance of intestinal immune homeostasis (Leonardi et al., 2022; Maloy & Kullberg, 2008). Dysregulated Th17-mediated responses are frequently associated with pathological enteritis. For example, dextran sulfate sodium salt (DSS)-induced enteritis in grouper (*Epinephelus coioides*) involves marked up-regulation of pro-inflammatory mediators that trigger intestinal mucosal inflammation (Yu et al., 2024). Similarly, soybean meal-induced enteritis in hybrid yellow catfish (*Pelteobagrus fulvidraco* ♀×*P. vachelli* ♂) is characterized by extensive infiltration of inflammatory cells and granulocytes, with significant induction of *IL-1β* expression and a concomitant reduction in *IL-10* expression (Zhang et al., 2025b). Intestinal inflammation induced by *Aeromonas hydrophila* infection in juvenile blunt-snout bream (*Megalobrama amblycephala*) exhibits symptoms comparable to those observed in carp fed soybean meal (Xia et al., 2024). Collectively, these findings underscore the critical involvement of interleukins in regulating intestinal immune dynamics and highlight their central role in mediating the balance between protective immunity and pathological inflammation in fish enteritis.

Th17 cells function as critical mediators of mucosal inflammation by secreting interleukin 22 (IL-22), a multifunctional cytokine with central roles in immune regulation and tissue protection. In mammals, IL-22 signaling activates transcriptional programs associated with anti-inflammatory, mitogenic, proliferative, and anti-apoptotic responses, thereby supporting tissue and organ growth, remodeling, and repair while strengthening epithelial defense mechanisms against pathogen invasion (Keir et al., 2020). In teleost fish, IL-22 is predominantly expressed in mucosal tissues, particularly in the intestine and gills, and its production is strongly induced by pro-inflammatory stimuli such as lipopolysaccharides (LPS) and cytokines (Huo et al., 2019; Watanabe et al., 2023). In rainbow trout (*Oncorhynchus mykiss*), IL-22-producing cells localize to gill filaments and interbranchial lymphoid tissues, where they accumulate during *Aeromonas salmonicida* infection (Hu et al., 2019). The AHR/IL-22/STAT3 signaling axis has also emerged as a pivotal regulator of intestinal mucosal immunity, with IL-22 reported to regulate the expression of antimicrobial peptides (e.g., Reg-3γ) (Zheng et al., 2008) and tight junction proteins (e.g., Claudin) (Wang et al., 2017), thereby maintaining epithelial barrier integrity and microbiota homeostasis. In mandarin fish (*Siniperca chuatsi*), intestinal IL-22 up-regulates the expression of antimicrobial peptides such as hepcidin and liver-enriched antimicrobial peptide 2 (LEAP2) (Huo et al., 2021). In So-iny mullet (*Liza haematocheila*), intraperitoneal injection of recombinant IL-22 significantly boosts β-defensin expression in the intestine, improving survival during *Streptococcus dysgalactiae* infection (Qi et al., 2015). Despite these advances, knowledge regarding IL-22-mediated immune mechanisms in fish, particularly under environmental stressors such as hypoxia, remains limited.

The intestinal epithelium normally functions under a state of physiological hypoxia, a condition essential for maintaining epithelial metabolism and regulating intestinal mucosal barrier integrity. During enteritis, however, oxygen tension within IECs decreases sharply, creating a hypoxic microenvironment accompanied by excessive production of reactive oxygen species (ROS). These ROS regulate hypoxia-inducible factor 1α (HIF-1α) through multiple signaling pathways, triggering mucosal barrier-related regulatory mechanisms (Chen et al., 2023a). HIF-1α has been shown to drive the expression of pro-inflammatory cytokines via various routes, thereby contributing to the development of intestinal inflammation (Shi et al., 2023). In rheumatoid arthritis (RA), IL-33 stimulates synovial fibroblasts to secrete HIF-1α, establishing a HIF-1α/IL-33 feedback loop that exacerbates inflammatory responses (Hu et al., 2013). In teleosts, butyrate activates the STAT3/HIF-1α/IL-22 signaling cascade in turbot (*Scophthalmus maximus*) intestines, inducing autophagy and enhancing macrophage-mediated pathogen clearance (Zhang et al., 2023). However, the direct role of HIF-1α in regulating inflammatory factor expression to sustain intestinal homeostasis in fish remains insufficiently characterized.

Yellow catfish (*P. fulvidraco*) represents a commercially valuable freshwater species, yet intensive aquaculture practices often lead to localized hypoxia, increased pathogen exposure, and recurrent intestinal inflammation (Zhou et al., 2021). In mammals, IL-22 is recognized as a central regulator of mucosal immunity and inflammatory responses; however, its bioactivity in teleosts is not well understood. Given that the intestine serves as a primary target organ for hypoxia-induced stress responses, exploring the expression patterns and regulatory mechanisms of IL-22 in the intestine of yellow catfish under hypoxic stress is critical for the prevention and treatment of intestinal inflammation. This study examined the expression dynamics of *P. fulvidraco* IL-22 (*Pf_IL-22*) in intestinal tissues and primary IECs under hypoxic stress, evaluated the bioactivity of recombinant *Pf*_IL-22 (r*Pf*_IL-22) in modulating mRNA expression of inflammation-related genes, and characterized the interaction between HIF-1α and IL-22. The findings of this study uncover a previously unrecognized role of the HIF-1α/IL-22 axis in hypoxia-driven intestinal inflammation and provide mechanistic insights into mucosal immune regulation and potential strategies for mitigating inflammatory disease in teleost aquaculture.

MATERIALS AND METHODS

Fish and cell lines

Healthy yellow catfish (10.2±1.4 g) were obtained from the Nanjing Institute of Fisheries Science (Jiangsu, China). Fish were temporarily cultured in a recirculating water culture system in the lab at a temperature of 25.0±1.0°C and pH of 7.3±0.1 and fed twice daily (0900h and 1700h). The fish were given a two week acclimation period before the commencement of treatment. All animal experiments were conducted in accordance with the Institutional Animal and Use Committee of Nanjing Normal University and China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Human embryonic kidney cells (HEK-293T) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sangon Biotech, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 37°C in a humidified incubator provided with 5% CO_2.

Primary IEC isolation

Fish were anesthetized with eugenol (1:10 000) (purity 99%, Shanghai Reagent Corp, China) after fasting for 24 h prior to sampling. Intestinal tissues were longitudinally excised and

repeatedly rinsed with phosphate-buffered saline (PBS) containing penicillin-streptomycin (Beyotime, China). The tissues were minced into 1 mm^3 pieces and digested in 0.03% collagenase type Ⅰ and Ⅳ (Servicebio, China) in a T25 cell culture flask, followed by incubation in a shaker at 28°C and 100 r/min for 35 min. An equal volume of stopping solution (DMEM containing 1% penicillin-streptomycin and 10% FBS) was added to terminate digestion. The mixture was pipetted for 3 min and left to stand for 1 min to allow sedimentation. The supernatant was aspirated from the flask and passed through a 100 μm cell strainer (Biologix, China). The cell suspension was centrifuged for 5 min at 1 000 r/min at 28°C, and the pellet was washed several times with PBS/penicillin-streptomycin. Isolated IECs were seeded into collagen I-coated 12-well plates (Sigma-Aldrich, USA) at a density of 1.0×10^5 cells/mL and cultured with DMEM containing 18% FBS (Gibco, USA), 2% yellow catfish serum, 10 ng/mL epidermal growth factor (Sigma-Aldrich, USA), 1× ITS Media Supplement (Gibco, USA), 1× non-essential amino acid (Servicebio, China), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Beyotime, China). Cultures were incubated in a humidified atmosphere with 5% CO_2 at 28°C.

IL-22 sequence identification and phylogenetic analyses

The open reading frame (ORF) of the IL-22 gene in yellow catfish was identified using gene-specific primers (Supplementary Table S1) designed from transcriptomic data (Zhang et al., 2025a). Polymerase chain reaction (PCR) amplification was performed using 2× Phanta Max Master Mix (Vazyme, China), with the resulting products cloned into a pMD-19T Vector (Takara, Japan). Recombinant bacterial cultures were submitted to TSINGKE Biotech Co., Ltd. (China) for sequencing. The IL-22 nucleotide sequence was assembled and analyzed using DNAMAN v.8.0. The deduced amino acid sequence was used to predict functional domain architecture of *P. fulvidraco* IL-22 (*Pf*_IL-22) using the Simple Molecular Architecture Research Tool (SMART v.7.0) (http://smart.embl-heidelberg.de/). The theoretical isoelectric point and molecular weight were calculated using ExPASy (https://web.expasy.org/compute_pi/), and amino acid translation was performed using the ExPASy translate server (https://web.expasy.org/translate/). Multiple sequence alignment of IL-22 amino acid sequences was performed using ClustalW in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Signal peptides were predicted using the SignalP v5.0 server (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree was constructed using the neighbor-joining method, with 10 000 replicates to obtain bootstrap values.

Modulation of gene expression *in vivo* and *in vitro* under acute hypoxia exposure

Ninety yellow catfish were evenly distributed into three aquaria equipped with aquaculture water-recycling biofilters, with 30 individuals per tank to establish three parallel experimental units. The overall experimental setup and sampling process are illustrated in Supplementary Figure S1. Prior to the experiment, three fish were randomly selected from each aquarium to obtain baseline samples, and at each subsequent time point, three fish were randomly sampled from each of the three tanks. Following dissection, intestinal tissues from these fish were pooled equally to form a single composite sample per tank. In total, three pooled samples were prepared and designated as the hypoxia 0-hour (0 h) group. To induce hypoxia, the aeration system and water intake were shut down, and the water surface was sealed with transparent film. Nitrogen gas was introduced to reduce dissolved oxygen (DO) levels to 0.85±0.05 mg/L, a concentration determined from previous studies (Zhang et al., 2025a). DO levels were monitored every 30 min and maintained at the target concentration by adjusting the oxygen pump or supplementing nitrogen gas as needed. Fish were sampled at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h post-treatment using the same sampling and pooling methods as the 0 h group. All intestinal samples were immediately snap-frozen in liquid nitrogen and stored at −80℃ for RNA extraction, while remaining tissues were fixed in 4% paraformaldehyde (PFA) for histological analyses. For *Pf_IL-22* tissue distribution profiling, nine healthy fish were used, with three individuals pooled per composite sample. Ten tissues and organs were collected: liver, head kidney, spleen, brain, heart, hindgut, gill, muscle, blood, and skin.

Primary IECs were isolated following previously described methods and seeded into 12-well plates at a density of 1 mL per well. Cells were incubated for 48 h prior to treatment. For the HIF-1α overexpression assay, 1 mmol/L dimethyloxallyl glycine (DMOG) (Aladdin, China) was added to the culture medium, with dimethyl sulfoxide (DMSO) (Solarbio, China) serving as the control. Cells were harvested after 12 h of treatment to assess gene overexpression efficiency.

Histological observation

After 24 h of fixation, intestinal tissues were dehydrated through a graded methanol series (30% to 100%), rinsed with ethanol and xylene, embedded in paraffin, and sectioned into slices (5 μm). After staining with hematoxylin and eosin (H&E), the sections were observed and imaged using a Nikon Ti-*E*-A1R light microscope (Tokyo, Japan).

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from intestinal tissues using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocols. RNA quality was evaluated by agarose gel electrophoresis, and RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). First-strand cDNA was synthesized from 500 ng of total RNA per sample using Hifair® Ⅲ 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen, China) according to the manufacturer's instructions.

The *β-actin* gene of yellow catfish was used as the internal reference for normalization of gene expression. Primer sequences used for RT-qPCR are listed in Supplementary Table S1. RT-qPCR was performed in triplicate in a 20 μL reaction volume containing 10 μL of Hieff® qPCR SYBR Green Master Mix (No Rox) (Yeasen, China), 2 μL of cDNA (10-fold dilution of template), 0.4 μL each of forward and reverse primers (10 μmol/L), and 7.2 μL of distilled water. Amplification was carried out on a LightCycler 480 instrument (Roche Diagnostics, Switzerland) with the following program: 95℃ for 5 min, followed by 40 cycles of amplification at 95°C for 10 s and 60°C for 30 s, with melting curve analysis at 95℃ for 15 s to confirm specificity. Relative gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method.

Recombinant expression of *Pf*_IL-22 (r*Pf*_IL-22) and polyclonal antibody preparation

A fragment of the *Pf_IL-22* gene lacking the predicted signal peptide was cloned into the pET-32a vector for prokaryotic

expression. The recombinant Pf_IL-22 protein was refolded, concentrated using PEG-20000 (Solarbio, China), and stored at −80°C. Protein concentration was quantified using the Bradford protein assay (Jiancheng Bioengineering, China). Rabbit polyclonal antibodies against r*Pf*_IL-22 were generated by immunizing rabbits with r*Pf*_IL-22 at 14 d intervals for a total of three injections. Serum samples were collected from immunized animals 7 days after the final boost, and antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). The collected serum was subsequently purified and stored for further use.

Western blot analysis

Total protein was extracted from intestinal tissues using a commercial extraction kit (Jiangsu KeyGEN BioTECH, China). Protein samples were mixed with 5× loading buffer (Beyotime, China), boiled for 10 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation. The resolved proteins were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were then blocked in blocking buffer (10% (w/v) skim milk in TBST buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween 20, and pH 7.6)) at room temperature for 2 h, followed by three washes (10 min each) with TBST. Membranes were incubated for 12 h at 4℃ with primary antibodies, including mouse β-actin antibody (1:5 000; TransGen Biotech, China) and rabbit anti-r*Pf*_IL-22 (1:5 000). After three additional washes (10 min each) with TBST, membranes were incubated with goat anti-rabbit IgG (1:5 000; TransGen Biotech, China) or goat anti-mouse IgG (1:5 000; TransGen Biotech, China) secondary antibodies at room temperature for 2 h. Following three final TBST washes (10 min each), protein bands were visualized using a high-sensitivity chemiluminescence substrate kit (Tanon, China).

Modulation of gene expression by r*Pf*_IL-22 in IECs

Primary IECs were seeded into 12-well plates at a density of 1 mL per well and incubated for 48 h prior to treatment. Cells were then exposed to EB-1 buffer (control) or r*Pf*_IL-22 at concentrations of 5 ng/mL and 20 ng/mL for 12 h. Following treatment, cells were harvested, and total RNA was extracted as previously described. RT-qPCR was performed to assess the expression levels of pro-inflammatory cytokines potentially responsive to IL-22, including *IL-1β*, *IL-6*, *IL-8*, *IL-10*, *IL-21*, *IL-34*, *IL-17A/F1*, *TGF-β1*, *STAT3*, and *TNF-α*. The primers used for RT-qPCR are listed in Supplementary Table S1.

Transfections and dual-luciferase reporter assays

A 2 600 bp fragment upstream of the ATG start codon of *Pf*_IL-22, considered a key region of the *Pf*_IL-22 promoter, was amplified and cloned in the pGL3- basic vector to generate a pGL3-HRE reporter construct. A HIF-1α transcription factor plasmid (pcDNA-HIF-1α) was constructed to enable HIF-1α overexpression. A mutant reporter plasmid (pGL3-HRE-Mut), in which the predicted HIF-1α-binding site was disrupted, was generated using a QuickMutation™ Site-Directed Mutagenesis Kit (Beyotime, China). Prior to transient transfection, HEK293T cells were seeded into 24-well plates at a density of 1.5×10^5 cells/well and cultured for 24 h until reaching 70%–80% convergence. Plasmids were transiently transfected into HEK293T cells using Lipofectamine™ 3000 (Invitrogen, USA), following the manufacturer's protocols. Equimolar amounts of reporter plasmids were co-transfected with 20 ng of pRL-Thymidine Kinase (pRL-TK) plasmid as an internal control, using Opti-MEM (Invitrogen, USA) as the transfection medium. At 48 h post-transfection, cells were stimulated with or without 1 mM DMOG for 30 min, harvested, and subjected to dual-luciferase assays using the Dual-Luciferase Reporter Assay System (Promega, USA). Relative luciferase activity was determined as the firefly-to-*Renilla* luciferase ratio. Results were normalized to the control reporter pGL3-Basic. All experiments were performed in triplicate and measured at least three times.

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed to evaluate the binding of HIF-1α to the predicted hypoxia response elements (HREs) within the yellow catfish IL-22 promoter. Briefly, biotin-labeled and unlabeled double-stranded DNA probes containing the putative HRE were synthesized, along with biotin-labeled probes harboring mutated HIF-1α-binding sites, using a Chemiluminescent EMSA Kit (Beyotime, China). Nuclear proteins were extracted from the yellow catfish intestinal tissues using a Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN, China). Reaction mixtures containing 10 μg of nuclear protein, 100 nmol/L biotin-labeled probes, and 1× binding buffer were incubated for 20 min at room temperature. For competitive binding assays, a 100-fold molar excess of unlabeled or mutated probe was added to the nuclear extracts prior to the addition of labeled probes. Protein-DNA complexes were resolved on 4% polyacrylamide gels in 0.5× TBE via electrophoresis at 100 V for 90 min, followed by transfer to nylon membranes. Biotin-labeled DNA was detected using the chemiluminescent EMSA kit. All oligonucleotide sequences used for EMSA are listed in Supplementary Table S1.

RNA interference (RNAi) *in vivo*

Pf_HIF-1α knockdown was performed using small interfering RNA (siRNA) following previously described protocols (Zhang et al., 2024). Four candidate siRNAs targeting *Pf_HIF-1α* were designed using the GenScript online tool (https://www.genscript.com/tools/sirna-target-finder). The corresponding sequences were amplified by PCR and inserted into the siRNA expression vector pRNAT-CMV3.1 (GenScript, USA) to generate plasmids p*Pf*_HIF-1αsi-1, p*Pf*_HIF-1αsi-2, p*Pf*_HIF-1αsi-3, and p*Pf*_HIF-1αsi-4. Each siRNA plasmid (20 μg/fish) or PBS (control) was intramuscularly injected into groups of yellow catfish (n=5; 9.50±1.44 g) to detect interference efficiency. At 48 h post-injection, the expression level of *Pf_HIF-1α* in the intestine was detected by RT-qPCR, as described above. The plasmid showing the strongest knockdown efficiency was selected and designated as p*Pf*_HIF-1αsi. Primer sequences used are listed in Supplementary Table S1.

For *Pf*_HIF-1α knockdown, yellow catfish were randomly divided into two groups (n=20) and injected intramuscularly with p*Pf*_HIF-1αsi or p*Pf*_HIF-1αsiC (control). At 48 h post-injection, fish were exposed to acute hypoxia, as described in Section 2.4. Hypoxia exposure lasted 96 h, during which the interfering plasmid was re-administered every 48 h. Intestinal IL-22 expression levels were assessed by RT-qPCR after 48 h of acute hypoxia. Midgut tissues from wild-type and IL-22 knockdown fish under normoxia and after 96 h of hypoxia were harvested, processed for H&E staining, and examined histologically. Intestinal villus height was measured to evaluate structural alterations.

Transcriptomic analysis of *IL-22* knockdown under hypoxic stress

The p*Pf*_IL-22si interference plasmid was constructed using the same method described above to generate p*Pf*_IL-22si. For *Pf*_IL-22 knockdown, yellow catfish were randomly assigned to two groups (n=20) and intramuscularly injected with either p*Pf*_IL-22si or p*Pf_IL-22*siC (control). Following 48 h of normal culture and an additional 48 h of acute hypoxia exposure, intestinal tissues were collected for transcriptomic analysis (three pooled samples per group, each consisting of tissues from two fish). Total RNA was extracted, and six cDNA libraries were constructed and sequenced by Novogene Co., Ltd. (China) on the Illumina NovaSeq 6000 platform (USA). Quality control and data processing were performed following the same procedures as described in our previous study (Zhang et al., 2025a).

Statistical analysis

All statistical analyses were performed using SPSS v.26.0. Western blot band intensities were quantified, and confocal images were processed using ImageJ software. Data are presented as the mean±standard deviation (SD) of three independent experiments with three technical replicates for each experiment. Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) *post-hoc* test. Significance and high significance were accepted at P<0.05 and P<0.01, respectively.

RESULTS

Characterization of *Pf*_IL-22 cDNA and protein sequences

The ORF of *Pf*_IL-22 spanned 546 bp, encoding a peptide of 181 amino acids, with a predicted molecular weight of 44 966.97 Da and an isoelectric point of 5.15 (Figure 1A). A putative signal peptide comprising 26 amino acids was identified. Based on structural analysis, the *Pf*_IL-22 protein included an IL-22 domain (29–175 amino acids (aa)) and two N-glycosylation sites (Figure 1B). Multiple sequence alignment revealed four highly conserved cysteine residues forming two intramolecular disulfide bonds, a characteristic feature among teleosts (Figure 1C). Synteny analysis demonstrated strong conservation of the genomic architecture surrounding IL-22, showing high similarity with the zebrafish genome, including the presence of fish-specific *IFN-γ rel* genes downstream (Figure 1D). Notably, this conserved syntenic organization extends to humans and other species, with *rap1b* and *mdm1* genes consistently located upstream of *Pf_IL-22*, and *IL-26* and *IFN-γ* positioned downstream. Phylogenetic reconstruction using the neighbor-joining method revealed that *Pf_IL-22* clustered closely with its orthologs from *P. vachelli* and *Ictalurus punctatus*, forming a distinct clade that is sister to the lineage containing *Ctenopharyngodon idella* and *Danio rerio* IL-22 orthologs (Figure 1E). This phylogenetic topology highlights the evolutionary relatedness of these teleost species and reflects the high degree of conservation in IL-22 gene sequences across vertebrates.

Production and purification of r*Pf*_IL-22

The nucleotide sequence of the *Pf*_IL-22 ORF was validated through bioinformatic analyses based on the yellow catfish genome database. Subsequently, the pET32a-IL-22 prokaryotic expression plasmid was constructed for the expression of recombinant r*Pf*_IL-22 in *Escherichia coli*. SDS-PAGE and western blot analysis revealed that the molecular weight of r*Pf*_IL-22 was approximately 35.5 kDa (Supplementary Figure S2), consistent with predictions based on sequence structure analysis.

Construction of an intestinal hypoxia model in yellow catfish

An intestinal hypoxia model was established in yellow catfish to evaluate physiological and molecular responses to acute oxygen deprivation. Histopathological examination revealed that 72 h of acute hypoxic exposure induced significant structural abnormalities in midgut tissues compared with the control group. The intestinal epithelium exhibited marked disorganization of single-layer columnar cells, loosening of the lamina propria, increased vacuolation, reduced goblet cell density, and extensive villus erosion (Supplementary Figure S3A). RT-qPCR analysis of hypoxia-responsive genes demonstrated robust activation of the HIF pathway under hypoxic stress. Expression of HIF-1α increased significantly, peaking at 24 h before gradually declining (P<0.05) (Supplementary Figure S3B). In contrast, FIH expression initially increased but declined thereafter (Supplementary Figure S3C). The reduction in FIH at 24 h, combined with its role as an inhibitor of HIF-1α, indicates enhanced HIF-1α activation at this time point. Concurrently, VHL remained highly expressed under hypoxic conditions (P<0.05) (Supplementary Figure S3D). HIF-2α expression showed minimal variation during the early stages of hypoxia (0–12 h) (Supplementary Figure S3E) but increased significantly at 24 h, reaching its peak at 48 h (P<0.05). Collectively, these findings indicate that acute hypoxic stress strongly activates the intestinal HIF signaling cascade in yellow catfish, initiating transcriptional reprogramming and inflammatory responses.

Expression dynamics of *Pf*_IL-22 and cytokine genes under hypoxic stress

*Pf*_IL-22 transcript distribution was assessed across 10 distinct tissues in yellow catfish. RT-qPCR analysis detected *Pf*_IL-22 mRNA expression in all tested tissues (Supplementary Figure S4), with notably higher levels in the gill, hindgut, and skin—mucosal sites associated with immune defense—suggesting a potentially significant role for *Pf*_IL-22 in mucosal immunity. In contrast, relatively low transcript abundance was observed in the head kidney, heart, blood, and liver.

To investigate responses to hypoxic stress, temporal expression profiles of *Pf_IL-22* and related cytokines were assessed in intestinal tissues following 72 h of acute hypoxia (Figure 2). *Pf*_IL-22 levels showed a modest increase during the first 6 h of hypoxia, followed by a sharp increase at 24 h (P<0.05), reaching approximately 17-fold higher than control levels, before progressively declining to baseline by 72 h (P<0.05). Similar expression patterns were observed for *IL-22RA*, *IL-22BP*, *IL-10*, *IL-17A/F1*, *TGF-β1*, *STAT3*, and *AHR*, suggesting coordinated transcriptional regulation among these cytokines. In contrast, *IL-1β* displayed sustained high expression 6 h post-hypoxia (P<0.05).

Given these transcriptional trends, HIF-1α expression was further investigated to assess potential regulatory interactions with IL-22. Acute hypoxia significantly up-regulated both HIF-1α and IL-22 transcripts. Subsequent examination confirmed a correlation between HIF-1α and IL-22 expression at the protein level (Figure 3). Western blotting revealed that intestinal HIF-1α expression initially increased under hypoxia,

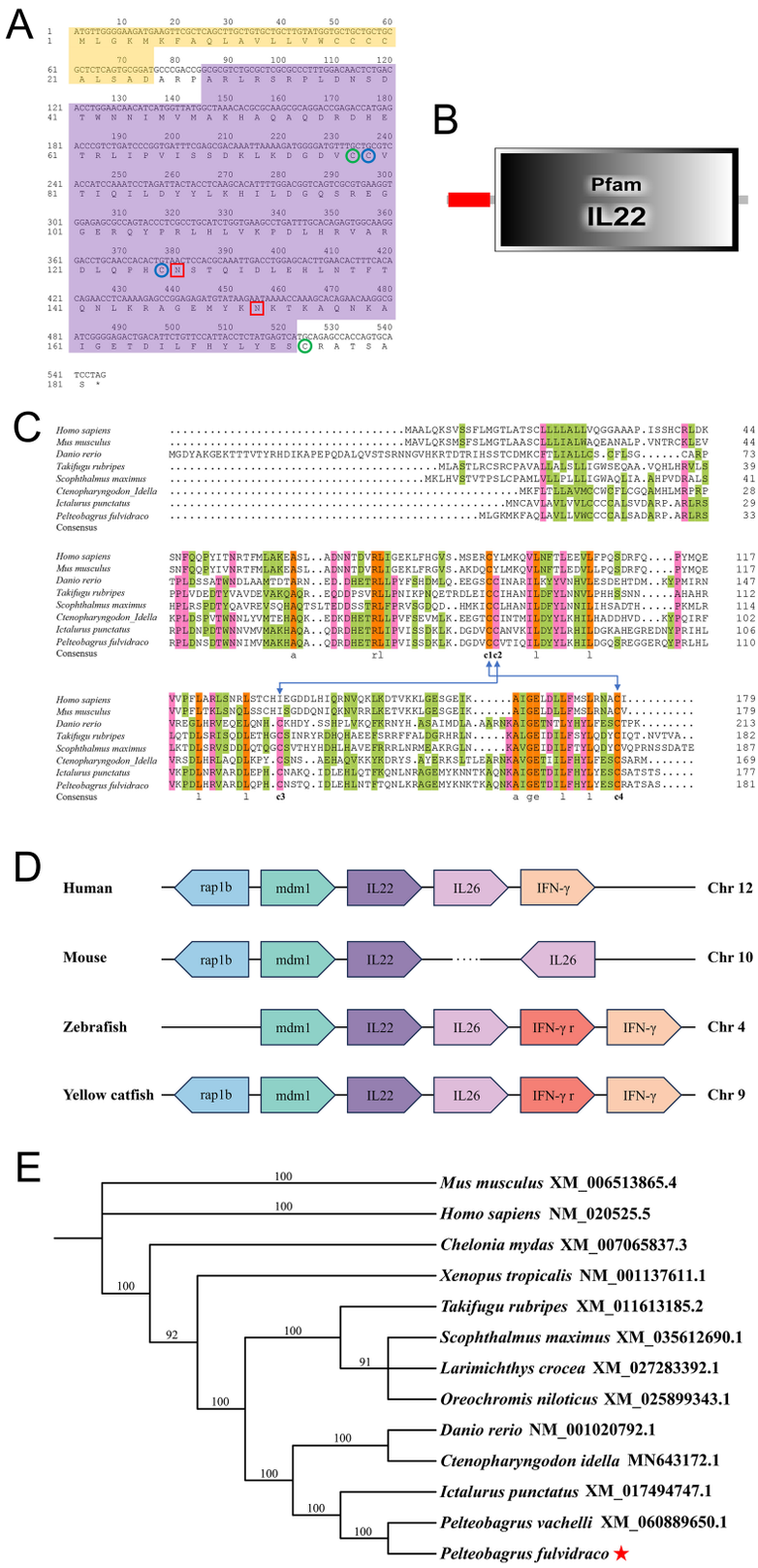

Figure 1 Characteristics of *Pf*_IL-22

A: ORF and deduced amino acid sequence of *Pf*_IL-22. Yellow box represents putative transmembrane region; purple region denotes IL-10 family domain; green and blue circles indicate cysteine residues; red square highlights N-glycosylation site. B: Protein structure of *Pf*_IL-22. Red box indicates transmembrane region. C: Multiple sequence alignment of teleost IL-22 amino acid sequences. Orange background indicates identical amino acid residues; green background indicates conserved amino acid residues; dot represents alignment gaps; C-C denotes disulfide bond. D: Synteny analysis of IL-22 across four species. Same color boxes represent orthologous genes between species; arrowhead represents direction of genes. E: Bayesian phylogenetic tree of IL-22 constructed using sequences from *P. fulvidraco* and other vertebrates, with node values representing bootstrap support; MCMC=200 000 generations.

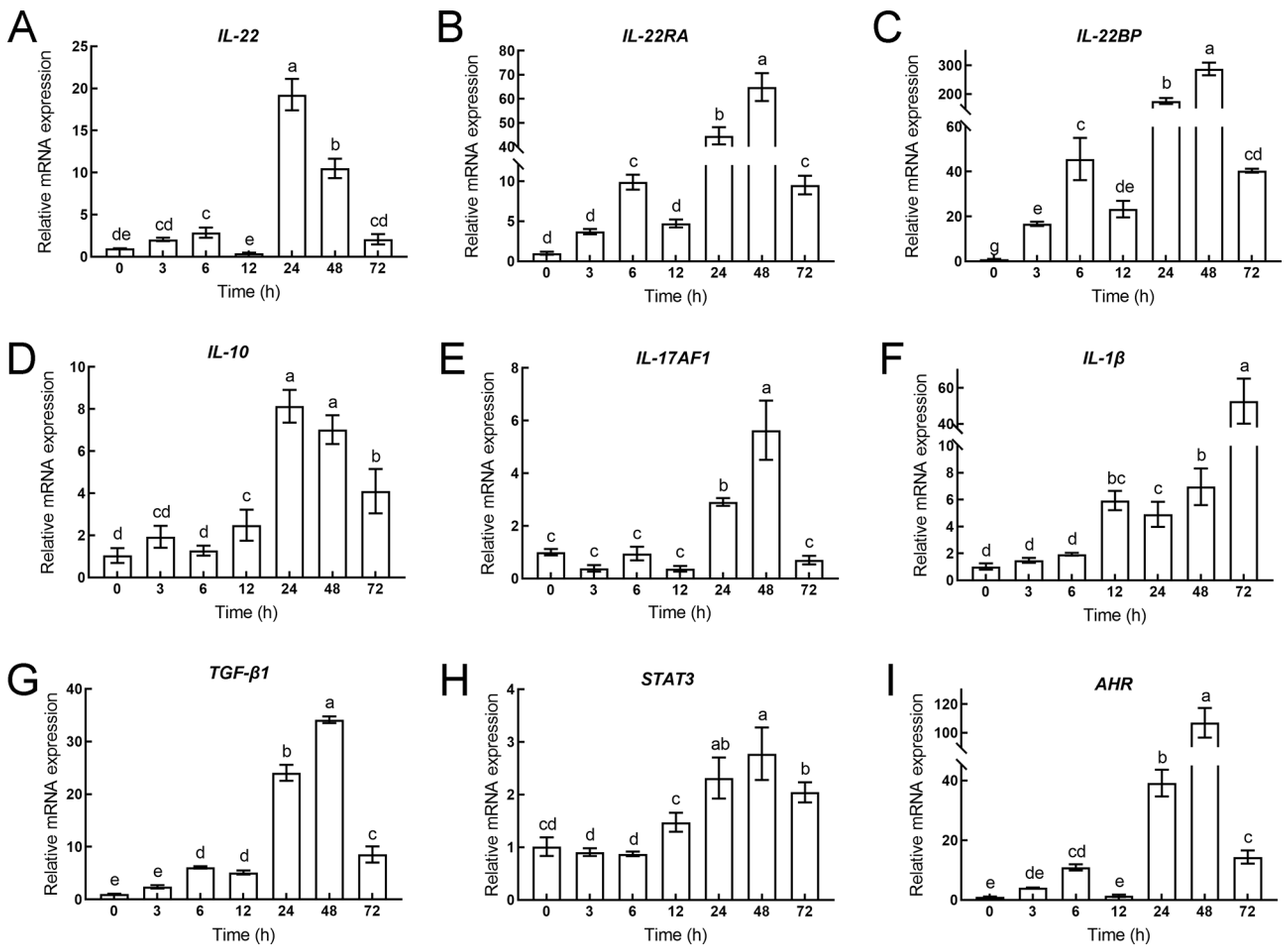

Figure 2 Temporal mRNA expression patterns of *Pf_IL-22* and related cytokines in the intestine of *P. fulvidraco* under hypoxic stress

Results are presented as mean±SD (*n*=3). Different lowercase letters indicate statistically significant differences (*P*<0.05).

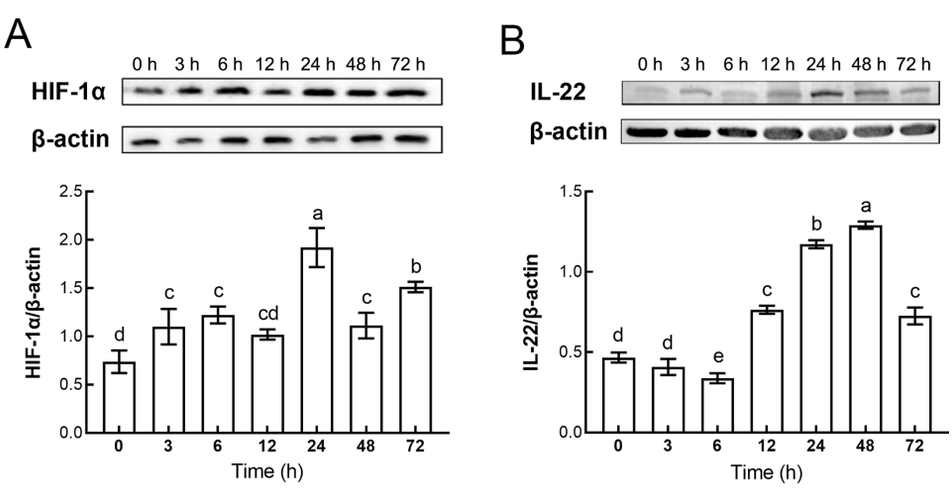

Figure 3 Western blot analysis of HIF-1α and IL-22 under hypoxic stress

Data are presented as mean±SD (*n*=3). Different lowercase letters indicate statistically significant differences (*P*<0.05).

peaking at 24 h (*P*<0.05) and declining thereafter. IL-22 protein expression exhibited a parallel temporal profile, aligning closely with HIF-1α dynamics across all measured time points. These concordant expression patterns suggest a potential mechanistic link between HIF-1α activation and IL-22 induction during hypoxic adaptation in yellow catfish.

Expression patterns of cytokine genes in IECs induced by r*Pf*_IL-22

The immunomodulatory effects of r*Pf*_IL-22 on inflammatory responses were evaluated in primary IECs derived from yellow catfish. As shown in Figure 4, exposure to 5 ng/mL r*Pf*_IL-22 inhibited the expression of most inflammatory cytokines, except *IL-34*, which exhibited a marked up-regulation. At 20 ng/mL, r*Pf*_IL-22 strongly induced *IL-1β*, *IL-6*, *IL-8*, *IL-17A/F1*, *IL-34*, and *TNF-α* expression, while significantly reducing *IL-10*, *IL-21*, *TGF-β1*, and *STAT3* levels, indicating a concentration-dependent bidirectional regulatory effect on cytokine production.

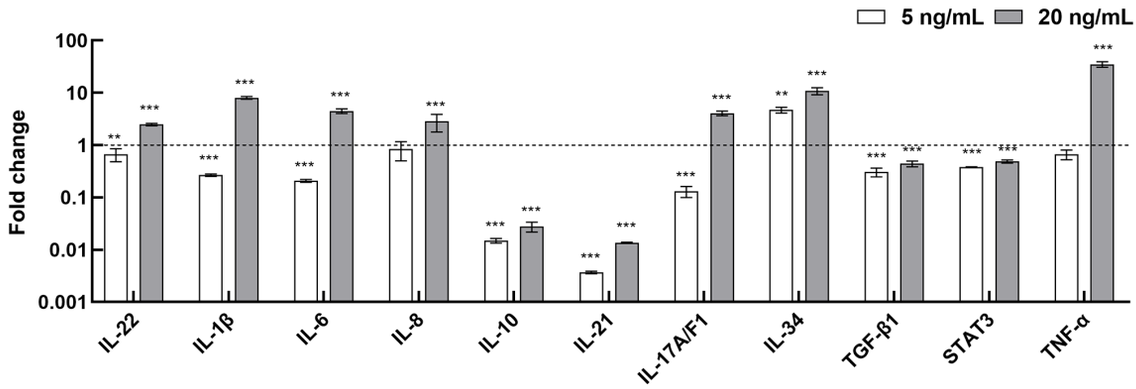

Figure 4 Effects of bacteria-derived r*Pf*_IL-22 on gene expression in primary intestinal epithelial cells (IECs)

Primary IECs were stimulated with r*Pf*_IL-22 for 12 h, and mRNA expression of cytokines was quantified by RT-qPCR. Data are presented as mean±SD (*n*=3). "*" indicates statistically significant differences (**: $P<0.01$; ***: $P<0.001$).

Expression analysis of *Pf*_IL-22 and cytokine genes in IECs following DMOG treatment

To investigate the regulation of IL-22 under hypoxia-mimicking conditions, IECs were treated with 1 mM DMOG, a HIF prolyl hydroxylase inhibitor and potent activator of HIF signaling, for 12 h (Figure 5A). Compared with DMSO-treated controls, DMOG significantly enhanced HIF-1α expression ($P<0.05$) (Figure 5B). This activation was accompanied by a two-fold increase in IL-22 transcript levels, suggesting a regulatory link between HIF-1α and IL-22. Furthermore, DMOG treatment markedly up-regulated *IL-22BP*, *IL-22RA1*, *IL-10*, *IL-1β*, *TGF-β1*, and *STAT3* expression in IECs ($P<0.05$) (Figure 5C). In contrast, *IL-17A/F1* and *AHR* expression showed a non-significant increase ($P>0.05$). These findings are consistent with *in vivo* observations, demonstrating that HIF-1α activation promotes IL-22 up-regulation and modulates downstream cytokine networks.

HIF-1α triggers transcriptional activation of *Pf*_IL-22 under hypoxic stress

To further investigate the regulatory role of HIF-1α on IL-22 gene expression, a 2 600 bp promoter sequence upstream of the IL-22 gene was initially cloned and analyzed. A putative HRE was identified between −1 940 to −1 947 bp (Figure 5D), suggesting a potential role for HIF-1α in modulating *Pf_IL-22* expression through direct HRE binding. Functional validation was performed using a dual-luciferase reporter system combined with site-directed mutagenesis. Three constructs were generated, including pGL3-HRE, pGL3-HRE-Mut, and pcDNA-HIF-1α expression vector. As shown in Figure 5E, co-transfection of pGL3-HRE with pcDNA-HIF-1α into HEK293T cells induced a significant increase in luciferase activity compared with the control group (pGL3-HRE+pcDNA-3.1). Treatment with DMOG further amplified this induction ($P<0.05$), indicating that DMOG enhances HIF-1α binding and transcriptional activation of the IL-22 promoter. In contrast to the pGL3-HRE+pcDNA-HIF-1α group, pGL3-HRE-Mut+pcDNA-HIF-1α showed a significant reduction in luciferase activity ($P<0.05$), comparable to that observed in the pGL3-HRE-Mut+pcDNA-3.1 group ($P>0.05$). Following DMOG stimulation, luciferase activity in the pGL3-HRE-Mut+pcDNA-HIF-1α group was unchanged, showing no significant difference from baseline ($P>0.05$). These findings suggest that HIF-1α regulates IL-22 transcription through direct interaction with the HRE in its promoter region.

To confirm HIF-1α binding specificity, an electrophoretic mobility shift assay (EMSA) was performed using biotin-labeled probes corresponding to the predicted HRE within the IL-22 promoter (Figure 5F). Nucleoproteins extracted from yellow catfish intestinal tissue formed distinct DNA-protein complexes with wild-type probes, demonstrating direct interaction between HIF-1α and the intact HRE. This binding was competitively disrupted by excess unlabeled wild-type probes but remained unaffected by unlabeled mutant probes, confirming sequence specificity. Furthermore, acute hypoxia exposure enhanced the formation of HIF-1α–HRE complexes, supporting a hypoxia-responsive mechanism of transcriptional regulation.

To further validate the role of HIF-1α in regulating *Pf*_IL-22 expression *in vivo*, HIF-1α knockdown was performed in *P. fulvidraco* using the p*Pf*_HIF-1αsi-interference construct. RT-qPCR confirmed significant suppression of HIF-1α expression in intestinal tissue following treatment (Figure 6A). At 48 h post-administration, p*Pf*_HIF-1αsi-treated fish exhibited significantly reduced *Pf*_IL-22 expression compared with control fish (Figure 6B), establishing HIF-1α as a critical driver of hypoxia-induced P*f*_IL-22 transcription.

Effects of *Pf_IL-22* knockdown on intestinal immune responses under hypoxic stress

To evaluate the functional role of *Pf*_IL-22 in regulating intestinal immune responses of *P. fulvidraco*, *Pf_IL-22* RNAi and transcriptomic analysis were carried out. The silencing efficiency of *Pf*_IL-22 was confirmed (Supplementary Figure S5). Principal component analysis (PCA) revealed a distinct separation between the interference and control groups (Figure 7A), with the first principal component (PC1) accounting for 72.5% of the total variance. This separation verified the successful induction of hypoxic stress and demonstrated a distinct transcriptional response upon *Pf_IL-22* knockdown. Differential expression analysis identified 214 up-regulated genes and 297 down-regulated genes in the interference group compared with controls (Figure 7B). KEGG pathway enrichment analysis of these differentially expressed genes (DEGs) identified significant enrichment of immune-related pathways such as microRNAs in cancer, cytokine-cytokine receptor interaction, and Th17 cell differentiation, and cell cycle-related pathways such as cell cycle and DNA replication (Figure 7C). Immunogenetic interaction network mapping yielded similar results (Figure 7D), with the DEGs primarily involved in pathways such as cytokine-cytokine receptor interaction, Th17 cell differentiation, antigen processing and presentation, viral protein interaction with cytokines and cytokine receptors, and microRNAs in cancer.

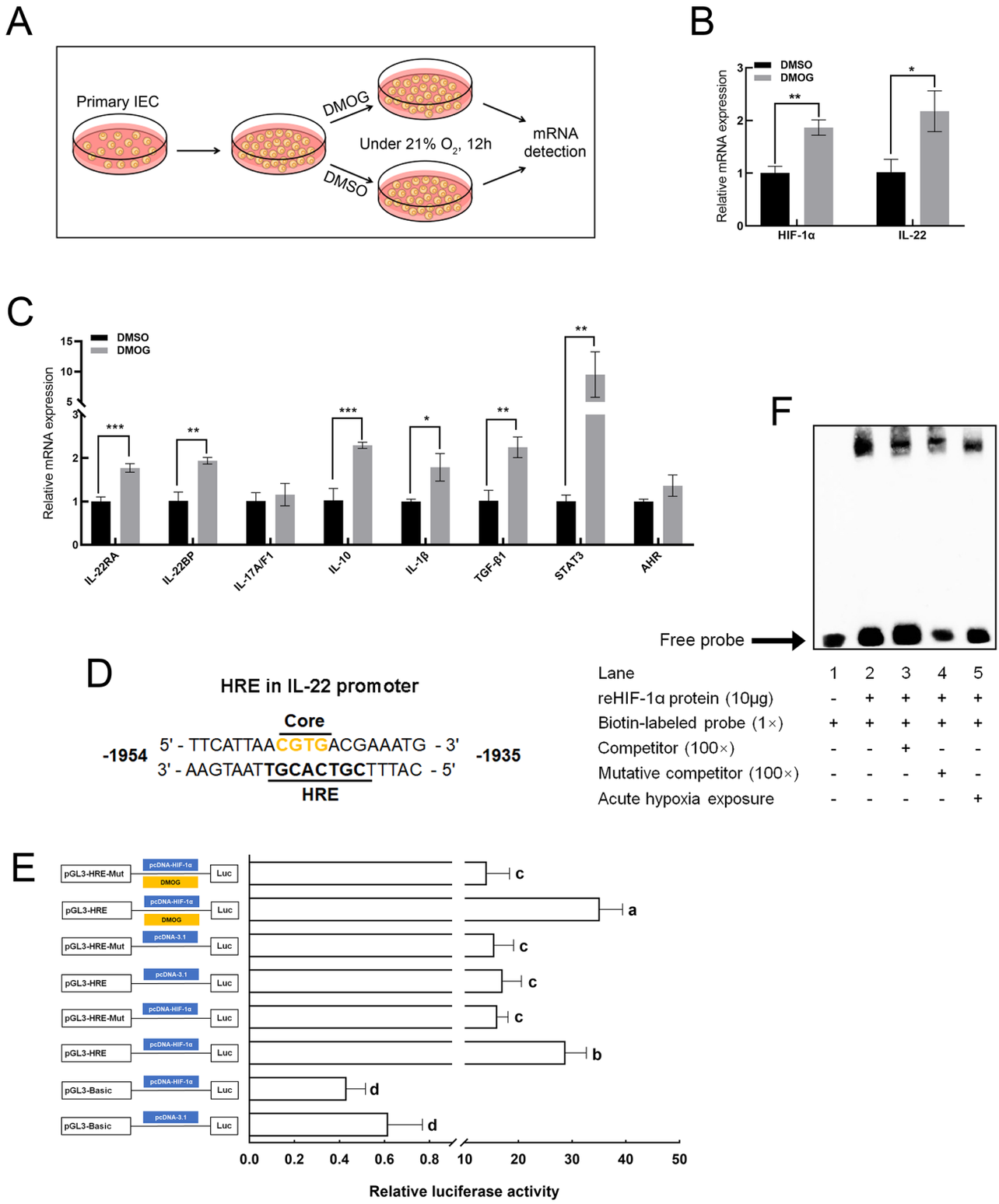

Figure 5 HIF-1α induces IL-22 production by binding to the hypoxia response element (HRE) in the IL-22 promoter region

A: Primary intestinal epithelial cells (IECs) were stimulated with DMOG or DMSO (control) for 12 h under normoxic conditions. B: Relative mRNA levels of HIF-1α and IL-22 in primary IECs. C: mRNA expression patterns of representative pro- and anti-inflammatory genes in IECs. RT-qPCR data are presented as mean±SD (n=3). "*" indicates statistically significant differences (*: P<0.05; **: P<0.01; ***: P<0.001). D: HRE binding sequence located between −1 940 bp and −1 947 bp of the *Pf*_IL-22 promoter region in yellow catfish. E: Site-directed mutagenesis analysis of HIF-1α binding sites in pGl3-*Pf*_IL-22 vectors. F: Electrophoretic mobility-shift assay (EMSA) of putative HRE-binding sequences. 5′-biotin-labeled double-stranded oligomers were incubated with nuclear proteins. For competition and mutant competition assays, a 100-fold excess of competitor or mutant competitor oligomers was added. Dual-luciferase reporter assay data are presented as mean±SD (n=6). Different lowercase letters indicate statistically significant differences (P<0.05).

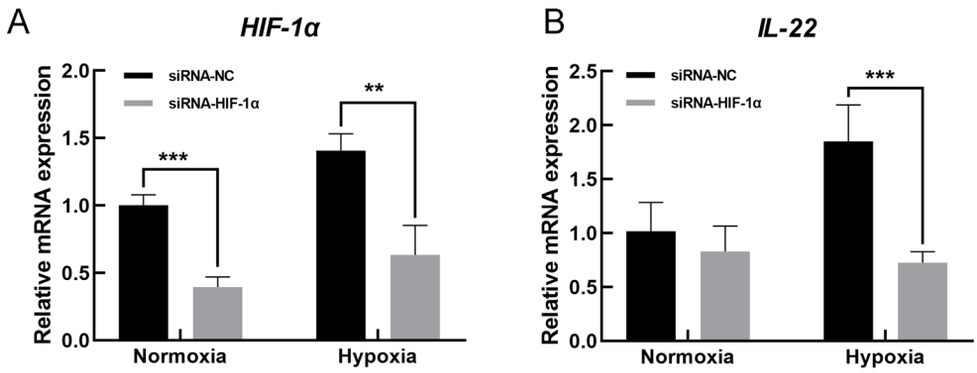

Figure 6 Effect of HIF-1α knockdown on hypoxic stress

Yellow catfish were pre-administered with p*Pf*_HIF-1αsi or p*Pf*_HIF-1αsiC (control) for 48 h, followed by 24 h exposure to hypoxia. Relative mRNA levels of *HIF-1α* and *IL-22* under normoxic and hypoxic conditions were detected in the intestine. Data are presented as mean±SD (*n*=3). "*" indicates statistically significant differences (**: *P*<0.01; ***: *P*<0.001).

Validation of selected genes confirmed a significant reduction in *Pf_IL-22* expression in the interference group, verifying the efficiency of RNAi (Figure 7E). HIF-1α expression remained unchanged, supporting earlier results that IL-22 does not influence HIF-1α regulation through negative feedback mechanisms. Similarly, STAT3 expression also showed no significant difference, contrasting with previous *in vivo* and *in vitro* findings. Further analysis of key genes in the Th17 cell differentiation pathway demonstrated significant down-regulation of nearly all genes except STAT3 (Figure 7F). Histopathological assessment revealed that IL-22 knockdown significantly reduced hypoxia-induced intestinal inflammation damage (Supplementary Figure S6), as evidenced by improved villus morphology and significantly increased villus length relative to wild-type individuals.

To validate the reliability of the transcriptomic data, RT-qPCR was performed on 12 DEGs associated with the Th17 pathway. The expression patterns obtained were highly consistent with transcriptomic trends (Supplementary Figure S7), demonstrating the accuracy and reliability of the sequencing results.

DISCUSSION

IL-22 plays a pivotal role in preserving intestinal immune homeostasis by strengthening epithelial barrier integrity and coordinating host defenses against pathogenic invasions (Wolk et al., 2004). Understanding the molecular regulation and functional roles of IL-22 in teleosts is imperative for advancing strategies to mitigate intestinal inflammation and immune dysfunction under environmental and physiological stress. Although IL-22 has been recognized as an important mediator in fish mucosal immunity, the regulatory mechanisms governing its expression under hypoxic stress remain poorly defined. This study comprehensively characterized IL-22 in yellow catfish based on its structural features, evolutionary relationships, tissue distribution, and expression profiles, and further assessed its regulatory relationship with HIF-1α in modulating intestinal inflammation. These findings expand the understanding of IL-22-mediated immune regulation in teleosts and highlight the HIF-1α/IL-22 axis as a potential modulator of intestinal inflammatory responses under hypoxia.

*Pf*_IL-22 exhibits conserved structural and functional features

The identified *Pf_IL-22* ORF, encoding a 181 aa protein with a predicted molecular weight of 20.7 kDa, exceeds the size reported for IL-22 homologs in blunt-snout bream (*M. amblycephala*) (168 aa) and grass carp (*C. idella*) (169 aa) (Wang et al., 2024; Yang et al., 2020b). Structural analysis revealed a predominantly hydrophilic profile, which may enhance solubility and facilitate interactions with other intracellular components (Bär et al., 2013). Four conserved cysteine residues were identified, with Cys78, Cys126, and Cys175 broadly conserved among vertebrates, while Cys79 represents a teleost-specific feature potentially linked to functional divergence. Comparative sequence analysis demonstrated high amino acid identity between *Pf*_IL-22 and other fish IL-22 orthologs, suggesting strong evolutionary conservation across teleosts.

Synteny mapping further revealed strong genomic conservation in the region surrounding *Pf_IL-22*, likely driven by evolutionary selective pressures (Marchi et al., 2021). Notably, *IFN-γ rel*, located downstream of *IL-22*, represents a unique interferon class found exclusively in teleosts with critical roles in antiviral defense and immune regulation (Ruan et al., 2017). The conserved genomic arrangement of IL-22, IL-26, and adjacent interferons suggests potential functional coordination in immune signaling. Supporting this, IL-22 has been reported to mitigate IFN-γ-mediated pulmonary inflammation (Pennino et al., 2013), implying a broader regulatory interplay among these cytokines. These findings indicate that IL-22 may act in concert with interferons to modulate immune responses in yellow catfish, highlighting a potentially important but unexplored regulatory network. Phylogenetic analysis revealed that *Pf_IL-22* shares strong sequence identity and evolutionary conservation with IL-22 orthologs from other teleosts, confirming accurate gene identification and supporting the likelihood of conserved functional roles across species.

Tissue distribution analysis revealed that *Pf*_IL-22 expression was highest in the gills, followed by the midgut, skin, and other mucosal tissues, suggesting an important role in mucosal immune defense. In teleosts, mucosal immune tissues enriched with B cells, T cells, and immunoglobulins are essential for pathogen clearance and maintenance of mucosal homeostasis (Yu et al., 2020). Previous studies on yellow catfish have also reported high IL-22 expression in the fin and other mucosal tissues, including the gills and midgut, consistent with these findings (Jiang et al., 2018) Given this distribution pattern, our study focused on the immunological functions of *Pf*_IL-22 within the intestinal environment.

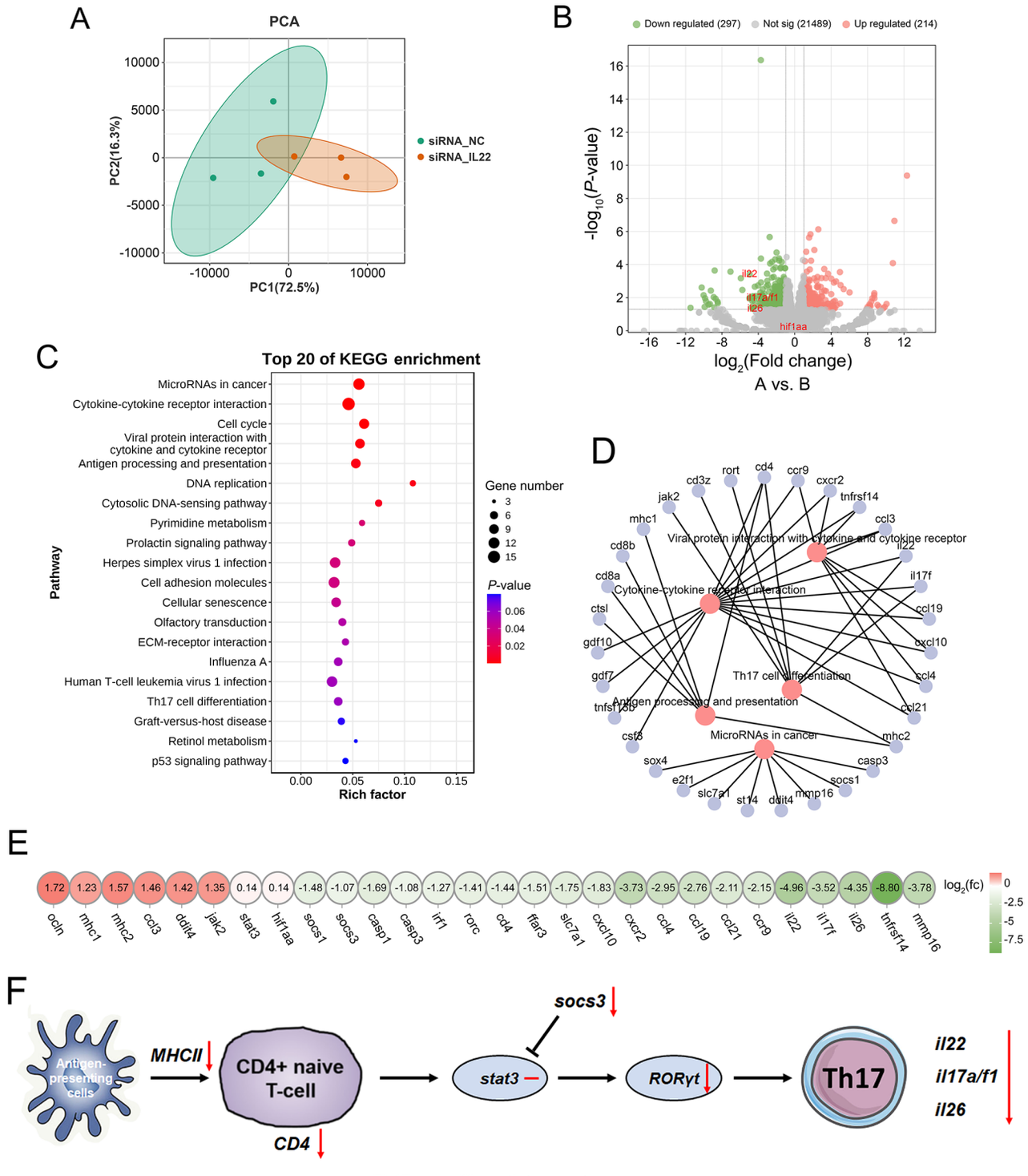

Figure 7 Effect of *Pf*_IL-22 knockdown on hypoxic stress based on transcriptomic analysis

A: Principal component analysis between p*Pf*_IL-22si and p*Pf*_IL-22siC (control). B: Volcano plot depicting DEGs between two groups. Gray dots indicate no significant difference, orange dots represent significantly up-regulated genes, and green dots represent significantly down-regulated genes. Four genes (*il22*, *il26*, *il17a/f1*, and *hif1aα*) are highlighted in red. C: KEGG pathway enrichment analysis of DEGs. D: Network showing interactions between hub genes and enriched pathways. E: Expression changes of immune-related genes after *Pf_IL-22* knockdown. F: Diagram illustrating changes in key Th17 pathway genes following IL-22 interference.

Expression dynamics of *Pf*_IL-22 under hypoxia exposure *in vivo*

Low DO can compromise intestinal structural integrity in fish, triggering inflammation and epithelial barrier dysfunction. IL-22 plays a crucial role in epithelial repair by promoting cell proliferation, regulating antimicrobial peptide production, and maintaining barrier homeostasis (Aujla et al., 2008; Wang et al., 2017). Hypoxic stress has been reported to induce IL-22 transcription (Budda et al., 2016). In this study, RT-qPCR analysis showed that *Pf*_IL-22 mRNA expression peaked at

24 h following hypoxia exposure, while IL-22 protein abundance reached its maximum at 48 h. The temporal lag between transcript and protein accumulation likely reflects post-transcriptional regulatory processes, indicating that mRNA levels may not directly predict protein secretion.

Histopathological analysis showed marked intestinal injury in yellow catfish under hypoxia, characterized by goblet cell swelling, vacuolation, and villous erosion. IL-22 is known to up-regulate genes involved in antimicrobial defense, facilitating secretion of antimicrobial peptides that protect intestinal tissues from microbial invasion (Liang et al., 2006). Therefore, the gradual increase in IL-22 protein levels may contribute to epithelial cell proliferation and restoration of barrier integrity disrupted by hypoxia. However, sustained overproduction of IL-22 has been linked to impaired intestinal homeostasis, with reports showing suppression of IEC proliferation, crypt loss, morphological changes, and reduced Lgr5+ stem cell potency in the small intestine, ultimately limiting stem cell differentiation into mature epithelial lineages (Zha et al., 2019). Thus, the decline in *Pf*_IL-22 expression after peak induction may represent a regulatory mechanism to down-regulate IL-22 and prevent excessive inflammation, suggesting a dual role of *Pf*_IL-22 in regulating protective immune responses and limiting excessive inflammation under hypoxic stress.

IL-22RA1 and IL-22BP exhibited expression profiles that paralleled IL-22, with a general increase over time and transient decreases at 12 h and 72 h post-hypoxia. In this study, the up-regulation of IL-22RA1 corresponded with rising IL-22 protein levels, suggesting enhanced receptor engagement during hypoxic stress. IL-22RA1 functions as the primary receptor for IL-22, forming a heterodimeric complex with IL-10R2 upon ligand binding to activate downstream signaling (Nikoopour et al., 2015). In contrast, IL-22BP acts as an endogenous inhibitor by binding IL-22 and preventing its interaction with IL-22RA1, thereby suppressing downstream responses. Previous studies have shown that IL-22 provides mucosal protection during acute inflammation but becomes detrimental if sustained at high levels during recovery. In IL-22BP$^{-/-}$ mice, excessive IL-22 availability promotes uncontrolled intestinal epithelial cell proliferation and increases tumorigenic potential (Huber et al., 2012). These findings, together with our observations, suggest that IL-22 in yellow catfish contributes to protection during the peak phase of hypoxic injury, whereas the subsequent rise in IL-22BP expression serves to modulate IL-22 activity, thereby preventing excessive intestinal cell proliferation and reducing the risk of inflammation or tumor development.

IL-22 functions within a complex cytokine network that integrates both pro-inflammatory and anti-inflammatory signals. For instance, IL-1β and IL-17 have been shown to induce IL-22 production in NK-22 cells (Cella et al., 2010), while IL-17A, IL-22, and TNF-α act synergistically to enhance keratinocyte activity (Guilloteau et al., 2010). IL-1β serves as a key pro-inflammatory mediator that activates and regulates immune responses across multiple cells, including T cells, B cells, neutrophils, and endothelial cells. In contrast, IL-10 functions as a critical immunoregulatory cytokine, suppressing inflammatory mediator release by monocytes and macrophages while promoting anti-inflammatory factor production (Wu et al., 2021). IL-17A and IL-17F, key drivers of inflammatory signaling, are essential for pathogen defense and maintenance of intestinal mucosal immunity (Okamura et al., 2020). In this study, the expression levels of *IL-1β* and *IL-10* increased with prolonged hypoxia, while IL-17A/F1, a homolog of IL-17A and IL-17F, also displayed marked up-regulation. Similar patterns have been reported in coho salmon (*Oncorhynchus kisutch*), where IL-1β and IL-10 expression in the spleen has been shown to increase under low DO conditions (Martínez et al., 2020). Together, these findings suggest that IL-22 may act in concert with other interleukins, amplifying its anti-inflammatory effects through synergistic interactions.

STAT3 and TGF-β1 are critical transcriptional regulators involved in diverse cellular processes, including differentiation, proliferation, apoptosis, and immune modulation. The IL-22 promoter in CD4$^+$ T cells contains binding sites for both STAT3 and AhR (Yeste et al., 2014), implicating these factors in the regulation of IL-22 expression. In turbot (*S. maximus*), butyrate promotes STAT3 phosphorylation in IECs, activating autophagy via the HIF-1α/IL-22 signaling pathway and enhancing host defense against invasive pathogens (Zhang et al., 2023). In this study, *STAT3* and *TGF-β1* exhibited comparable expression patterns under hypoxia, remaining largely unchanged during the early phase of hypoxia but showing marked up-regulation during the mid-phase, with peak levels observed at 48 h. These results suggest that the up-regulation of STAT3 and TGF-β1 may contribute to compensatory responses against hypoxia-induced damage by enhancing robust IL-22 production in intestinal cells. TGF-β has also been reported to promote IL-22 production in Th17 cells via AhR induction and PI3K-mediated signaling. AhR, a ligand-dependent transcription factor, plays a pivotal role in regulating processes such as cell development, inflammation, and immunity, particularly in the gut (Yeste et al., 2014). Supporting its relevance, oral administration of chitooligosaccharides (COS) has been shown to restore AhR/IL-22 signaling, increase MUC2 expression, repair the intestinal mucosal barrier, and alleviate colitis in mice (Wang et al., 2023). Consistently, in *P. fulvidraco* under hypoxic stress, intestinal *AhR* expression exhibited a consistent upward trend, peaking at 48 h, indicating that hypoxia may drive AhR up-regulation to facilitate IL-22 activation, thereby mitigating hypoxia-induced intestinal damage and promoting epithelial barrier repair.

Dual functions of HIF-1α/IL-22 axis in primary IECs under hypoxic stress

DMOG, a prolyl hydroxylase domain inhibitor, mimics hypoxic conditions by preventing HIF-1α degradation and promoting its nuclear accumulation (Chen et al., 2023b). In primary IECs, stimulation with 1 mM DMOG significantly increased *Pf_HIF-1α* expression, confirming successful activation of the HIF pathway. *Pf*_IL-22 also markedly increased under these conditions. Previous studies have demonstrated that HIF-1α enhances IL-22 transcription in CD4$^+$ T cells (Yang et al., 2020a). In this study, DMOG stimulation promoted HIF-1α nuclear accumulation in primary IECs, which was associated with a marked induction of IL-22 protein. Additionally, DMOG-treated IECs exhibited significant upregulation of *IL-22BP, IL-22RA1, IL-10, IL-1β, TGF-β1,* and *STAT3*, while *IL-17A/F1* and *AHR* showed non-significant upward trends. These results indicate that hypoxia-driven HIF-1α activation contributes to the regulation of IL-22 production and modulates a broader network of innate immune genes in yellow catfish.

Subsequently, the biological effects of r*Pf*_IL-22 on

immune-related gene expression in primary IECs were assessed. Results indicated that r*Pf*_IL-22 significantly induced the expression of multiple cytokines in IECs. While previous studies in grass carp primary head kidney leukocytes and CIK cells have reported that IL-22 predominantly regulates gene expression in leukocytes with limited influence on non-leukocytes (Yang et al., 2020b), the present findings indicated that IL-22 also exerts a notable regulatory effect on non-leukocyte populations. However, potential leukocyte-specific effects in yellow catfish were not investigated here, warranting further exploration. Moreover, the response to r*Pf*_IL-22 was dose-dependent: low-dose stimulation (5 ng/mL) produced minimal induction and even suppressed certain genes, whereas higher concentrations (20 ng/mL) robustly enhanced cytokine expression.

Previous studies have demonstrated that IL-22 performs dual and context-dependent functions: at moderate levels, it supports intestinal epithelial barrier integrity, while excessive expression can exert detrimental effects, including the promotion of colorectal carcinogenesis (Budda et al., 2016). In teleosts, IL-22 engages in complex crosstalk with both pro- and anti-inflammatory cytokines, paralleling observations in mammals. For instance, in grass carp (*C. idella*), IL-22 acts synergistically with IL-17A/F1 to enhance antimicrobial peptide production, such as β-defensin, within mucosal tissues, reflecting the cooperative roles of Th17-associated cytokines in mucosal defense (Yang et al., 2020b). Conversely, IL-22 also modulates anti-inflammatory pathways by inducing IL-10 and TGF-β1 expression in IECs, as observed in turbot (*S. maximus*) under bacterial challenge (Zhang et al., 2023). This dual functionality—amplifying pro-inflammatory signals for pathogen clearance and anti-inflammatory signals for tissue repair—appears evolutionarily conserved among teleosts. Thus, defining the optimal IL-22 concentration required to achieve effective immune protection without triggering pathological outcomes is essential. Notably, the present study revealed that IL-22 did not activate cellular responses via STAT3-mediated signaling in yellow catfish. Treatment with r*Pf*_IL-22 did not increase STAT3 expression, and silencing *Pf*_IL-22 similarly had no effect on STAT3 levels. This contrasts with observations in mammals (Yeste et al., 2014) and turbot (Zhang et al., 2023), where STAT3 functions as a central transcription factor downstream of IL-22.

HIF-1α activates IL-22 expression by directly binding to HREs

The hypoxic microenvironment of the gut induces the expression of several HIF-1α target genes in IECs, influencing their metabolism, barrier function, and survival (Fachi et al., 2024). As the regulatory subunit of the HIF-1 complex, HIF-1α modulates gene expression by binding to HREs in target promoters and has been implicated in the regulation of various $CD4^+$ T cell functions (Wang et al., 1995; Zhao et al., 2024). Using dual-luciferase reporter assays and EMSA, this study demonstrated that HIF-1α directly interacts with the IL-22 promoter in yellow catfish, establishing a mechanistic link between hypoxia signaling and IL-22 induction. Evidence from mammals supports this regulatory pathway; in mice, butyrate enhances the binding of HIF-1α to *IL-22* promoter HREs by increasing H3K9 acetylation and inhibiting H3K9 trimethylation (Sun et al., 2017). Similarly, in *S. maximus*, where butyrate activates the HIF-1α/IL-22 axis via type 3 innate lymphoid cells and STAT3-dependent signaling (Zhang et al., 2023). Notably, microbiota-derived short-chain fatty acids (SCFAs), particularly butyrate, play a critical role in sustaining physiological hypoxia in the gut by modulating epithelial oxygen metabolism. Butyrate activates peroxisome proliferator-activated receptor γ (PPARγ), enhancing mitochondrial β-oxidation and promoting oxygen consumption in IECs, which subsequently triggers HIF-1α stabilization and transcriptional activity (Byndloss et al., 2017; Kelly et al., 2015). In $CD4^+$ T cells and ILCs, butyrate treatment elevates HIF-1α abundance, while pharmacological or genetic inhibition of HIF-1α abolishes butyrate-induced IL-22 production, highlighting the critical role of HIF-1α in linking microbial metabolites to IL-22 regulation (Yang et al., 2020a). Hypoxia itself independently promotes IL-22 induction in $CD4^+$ T cells, and butyrate treatment under hypoxic conditions further enhances this response. This study provides the first evidence of a direct interaction between HIF-1α and IL-22 in teleosts. *In vivo* knockdown of HIF-1α significantly affected IL-22 expression under both normoxic and hypoxic conditions, indicating that both physiological and environmental hypoxia robustly activate the HIF-1α/IL-22 axis, playing a crucial role in maintaining intestinal immune homeostasis.

***Pf*_IL-22 exerts immune protection through the Th17 signaling pathway**

IL-22 gene knockdown in *P. fulvidraco* followed by RNA-seq of intestinal tissues under hypoxic stress identified key pathways and genes involved in the regulation of inflammation by IL-22. KEGG pathway enrichment analysis indicated that DEGs were predominantly enriched in immune- and inflammation-related pathways, including cytokine-cytokine receptor interaction, microRNA signaling, and Th17 signaling. The Th17 pathway plays a crucial role in host defense, tissue repair, and inflammatory disease pathogenesis (Amatya et al., 2017), prompting focused analysis of the role of this pathway in intestinal inflammatory responses. In mammals, Th17-associated cytokines such as IL-17A, IL-17F, and IL-22 contribute critically to mucosal barrier protection (Rutz et al., 2013). *Edwardsiella ictaluri* infection has been shown to reduce Th17 cell activity in the intestine of turbot, driving intestinal inflammation (Yang et al., 2024). IL-22 functions as a conserved marker for Th17 cell activity. In this study, IL-22 knockdown markedly inhibited Th17 signaling under acute hypoxia, with pronounced down-regulation of key genes including *IL-17A/F1* and *RORγt*. These findings indicate that IL-22 plays a pivotal role in sustaining hypoxia-induced intestinal inflammation. Notably, HIF-1α expression remained unchanged compared to controls, suggesting an absence of negative feedback regulation on IL-22 under hypoxia. Furthermore, STAT3 transcript levels were unaffected, likely reflecting its function via phosphorylation rather than transcriptional control, although potential compensatory mechanisms through alternative pathways warrant further investigation.

The gut microbiota, particularly SCFA-producing bacteria, plays a vital role in intestinal health. SCFAs serve as a critical link between the microbiome and intestinal immunity (Rooks & Garrett, 2016), with butyrate functioning via the FFAR3 receptor on ILC3 and $CD4^+$ T cells to induce AhR and HIF-1α expression, enhance accessibility of HIF-1α binding sites in the IL-22 promoter via histone modifications, and amplify IL-22 expression (Yang et al., 2020a). Consistent with this mechanism, IL-22 interference significantly reduced FFAR3

expression, implicating IL-22 in microbiota-driven immune modulation. SCFAs generated via microbial fermentation within the gut microbiota also exert immunomodulatory effects by engaging the FFAR3 receptor and inhibiting histone deacetylase activity, further enhancing IL-22 production by $CD4^+$ T cells and ILCs. Increased IL-22 secretion fortifies the intestinal epithelial barrier, thereby mitigating pathogen translocation and dampening inflammatory signaling pathways. Collectively, these mechanisms contribute to the maintenance of intestinal homeostasis in the host (Yang et al., 2020a). However, further comprehensive studies are needed to test these hypotheses and define the pathways through which the hypoxia-induced HIF-1α/IL-22 axis regulates ILC3 differentiation, maintenance, and migration, as well as its impact on microbiota composition.

CONCLUSION

This study demonstrated the roles of an IL-22 homolog in *P. fulvidraco* in defense against hypoxia. Hypoxia-induced activation of HIF-1α promoted its binding to HREs, leading to up-regulated IL-22 expression, which modulated downstream cytokine networks and mitigated intestinal inflammation. This regulatory process exhibited dual characteristics, with early-stage hypoxia exerting predominantly anti-inflammatory effects. Moreover, the Th17 signaling pathway was identified as a key mediator of IL-22-driven immunomodulation in the intestine. These findings clarify the molecular mechanisms by which the HIF-1α\IL-22 axis orchestrates immune responses during hypoxia, providing new insights into intestinal immune regulation and potential strategies for mitigating hypoxia-induced inflammatory damage in teleosts (Figure 8).

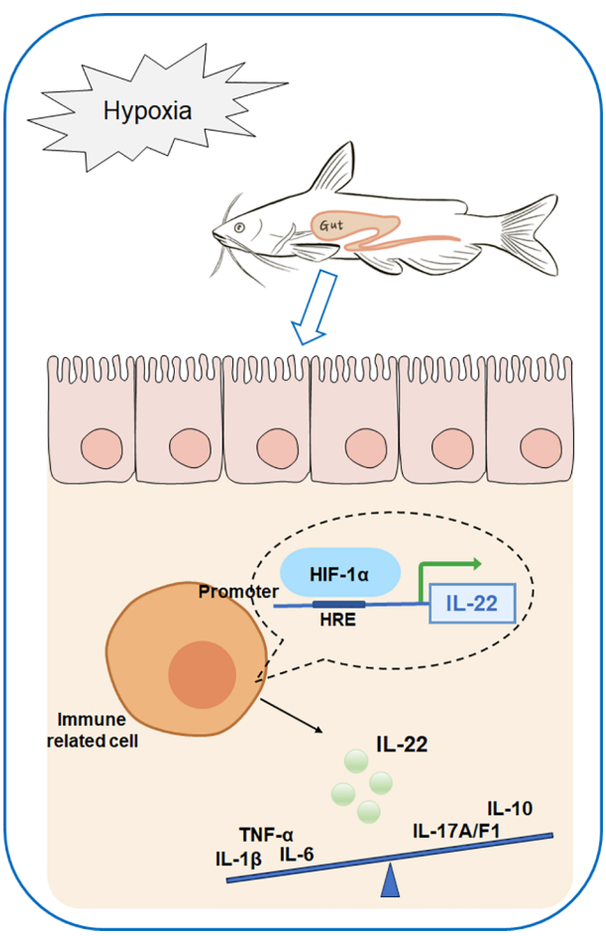

Figure 8 Schematic representation of HIF-1α/IL-22 signaling activation conferring resistance to hypoxia-induced intestinal inflammation in *P. fulvidraco*

Hypoxic stress activates HIF signaling, leading to HIF-1α binding to the HRE of IL-22 and inducing IL-22 production, which subsequently regulates the balance between pro-inflammatory and anti-inflammatory cytokines.

DATA AVAILABILITY

All sequencing data in the study were uploaded to the NCBI database under accession number SUB15212178/PRJNA1243326, National Genomics Data Center under GSA accession number CRA039430 (https://bigd.big.ac.cn/gsa/browse/CRA039430), and Science Data Bank under the link https://doi.org/10.57760/sciencedb.j00139.00191.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

S.W.Y. and K.Z. designed and conducted the research. H.Q.H., Y.B.D., and Z.A.Q. performed the experiments and analyzed the data. J.J., X.H.N., and K.Z. provided essential materials and technical support. H.Q.H., and K.Z. wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

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