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Identification and functional characterization of Interleukin-11 in goldfish (*Carassius auratus* L.)

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ABSTRACT

Interleukin-11 (IL-11) is a versatile cytokine that modulates cellular differentiation and proliferation in various cell types and tissues. In this study, IL-11 gene from goldfish (*Carassius auratus* L.) has been identified and characterized. Goldfish IL-11 (gfIL-11) has an open reading frame (ORF) that spans 591 base pairs (bp). The ORF encodes a precursor protein consisting of 196 amino acids (aa), which includes a 26 aa signal peptide and a conserved domain belonging to the IL-11 superfamily. Based on phylogenetic analysis, gfIL-11 was found to be closely related to other IL-11 homologues identified in various fish species. The gfIL-11 transcript exhibited varied expression levels across all the analyzed tissues, with the highest expression observed in the gill and spleen. Treatment of goldfish head kidney leukocytes (HKLs) with LPS and live *Aeromonas hydrophila*, increased gfIL-11 mRNA expression level. Recombinant gfIL-11 protein (rgIL-11) induced a dose-dependent production of TNF-α and IFNγ from goldfish HKLs. Furthermore, the administration of rgIL-11 to goldfish HKLs triggered an increase in the expression of various transcription factors such as MafB, cJun, GATA2, and Egr1, which play a vital role in the differentiation of myeloid precursors into macrophages and monocytes. Our findings provide evidence that IL-11 is a crucial cytokine that promotes cell proliferation, immune response, and differentiation across various hematopoietic lineages and stages of goldfish.

Introduction

Interleukin (IL)−6 is a family of cytokines that also includes IL-11, IL-27, IL-30, IL-31, oncostatin (OSM), leukemia inhibitory factor (LIF), cardiotrophin (CT-1), cardiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF), and neuropoitin (NP-1) [1]. These cytokines have been found to perform various functions such as bone formation, inflammation, immune cell activation, neuronal development and survival, hormone production, and hematopoiesis. While each cytokine serves a specific purpose, there is a significant amount of redundancy among family members due to their ability to signal through a GP130-containing complex on the cell surface [2]. All of these cytokines, except for IL-31, associate with the ubiquitously expressed signal transducer receptor glycoprotein GP130 as either a homodimer or heterodimer [1]. However, both IL-6 and IL-11 signal by the activation of a hexameric complex that includes the cytokine, the receptor for that cytokine (IL-6R or IL-11R), and GP130 [3,4].

IL-11 was first identified in a human fibroblast cell line [5], and has since been found in other species, including fish [6]. It is a 19 kDa non-glycosylated cationic protein made up of 178 aa, with a high proportion of leucine (~23 %) and proline (~12 %) [7]. The IL-11 precursor protein is cleaved before being released from cells and contains a 21 aa signal peptide sequence [8]. Mammalian IL-11 has multiple functions, such as controlling macrophage differentiation, speeding up megakaryocytopoiesis, and promoting the growth of "IL-6 dependent" plasmacytoma cells. The single-copy IL-11 molecule is widely expressed throughout mammalian organs and cell types. It protects the mucosa, promotes cell survival and proliferation, and is involved in immunological responses that contribute to inflammatory disorders and tumor development [5,9–12].

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Beside mammals, IL-11 genes have been found in amphibians and reptiles [13] and have recently been characterized for the first time in avian species [14]. The first non-mammalian IL-11 gene have been discovered in rainbow trout (*Oncorhynchus mykiss*) [6], subsequently it was also found in several species of bony fish including common carp (*Cyprinus carpio*) [15], Atlantic halibut (*Hippoglossus hippoglossus*) [16], Japanese flounder (*Paralichthys olivaceus*) [12], cobia (*Rachycentron canadum*) [17] and golden pompano (*Trachinotus ovatus*) [18], large yellow croaker (*Larimichthys crocea*) [19]. Furthermore, the *in silico* analysis of zebrafish genomes (*Danio rerio*), green-spotted pufferfish (*Tetraodon nigroviridis*) and tiger pufferfish (*Takifugu rubripes*) identified a duplicate fish IL-11 gene (IL-11a and IL-11b) [15]. Previous studies have consistently highlighted IL-11′s significant role in the immune response of fish. However, it is clear that almost no studies have been carried out on the leading role played by cytokines in general and IL-11, specifically in hematopoiesis in teleost. In contrast, research in the context of human hematopoiesis reveals that IL-11 collaborates with other cytokines within the hematopoietic microenvironment to enhance stem cell commitment to multilineage progenitors, drive their proliferation, and promote differentiation [20]. IL-11 exhibits synergistic effects with various early- and late-acting growth factors, thereby stimulating different stages and lineages of human hematopoiesis [21]. Additionally, the regulation of IL-11 in humans involves interactions with various transcription factors within its promoter region, such as signal transducer and activator of transcription 3 (STAT3) and activator protein 1 (AP-1) [9].

The hematopoietic properties of many growth factors in fish, including IL-11, remain largely unknown. Adipogenesis, protection of mucosal epithelia, and bone development are all influenced by IL-11, which is also important for regulating the proliferation and differentiation of hematopoietic progenitors. Although IL-11 has prominent roles in various physiological processes in mammalian models, the roles of IL-11 in hematopoiesis have not yet been characterized in fish. Goldfish (*Carassius auratus* L.) is an excellent model organism for the study of hematopoiesis and immunology in fish [22]. In this study, we identify and functionally characterize IL-11 in goldfish.

Materials and methods

Fish

Goldfish (*Carassius auratus* L.) were obtained from a local aquatic market in Ningbo and housed in the aquatic facilities of the Laboratory of Aquatic Animal Diseases and Immunology, University of Ningbo. Fish were acclimated for a minimum of 3 weeks, fed daily with pellets, and maintained at room temperature under a simulated natural photoperiod using a circulating water system. All fish were between 10 and 15 cm in length and were sedated with a solution of TMS (tricaine methane sulphonate) at 40 to 50 mg/L in water prior to handling. Protocol # NBU20210046 was approved by the Ningbo University Council of Animal Care, and all studies were carried out in conformity with these regulations.

Bacterial infection

To prepare the *A. hydrophila* strain for infection experiments, TSA plates were used to incubate the bacterial culture at 28 °C for 24 h. The culture was then transferred to TSB medium and shaken for 12 h at 28 °C. After centrifugation at 1000 *g* for 5 min at 4 °C, we washed the sediment thrice with phosphate-buffered saline (PBS). Two groups of fish were used for the infection assay: a control group and an experimental group. The control group received 100 μL of sterile PBS, while the experimental group received 100 μL of sterile PBS containing *A. hydrophila* intraperitoneally at a concentration of 1×10^8 CFU/mL.

RNA isolation and cDNA synthesis

Tissues including kidney (Ki), spleen (Sp), liver (Li), heart (He), brain (Br), muscle (Mu), intestine (In) and gills (Gi) were taken from 6 healthy goldfish. Following homogenization, tissues were extracted using Trizol reagent (Omega Biotech, China) to isolate RNA. After being checked for purity and concentration, RNA samples were stored at −80 °C and analyzed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcribing RNA and synthesizing first strand cDNA was carried out using a Prime-Script RT Reagent Kit with gDNA Eraser (Takara, Japan). Until needed, cDNA samples were kept at −20 °C.

Gene cloning and sequence analysis

The primers were designed according to the sequences obtained from the previously generated transcriptome database [23]. All primers employed in cloning and expression of gfIL-11 are provided in supplementary Table 1 (Table S1). For cDNA template preparation, we extracted RNA from goldfish kidney, followed by utilizing the PerfectStart Green qPCR SuperMix (TransGen, China). To amplify the product, we followed the thermocycling parameters consisting of an initial denaturation step at 95 °C for 2 min, followed by 30 PCR cycles at 95 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension of 72 °C for 12 min. Sequence study of gfIL-11 nucleotides and predicted amino acids, we used the NCBI BLAST program and the ExPASy server Molecular Biology (http://us.expasy.org). The Compute *p*I/Mw tool at ExPASy (http://www.expasy.ch/) was used to predict the molecular mass and isoelectric point of the putative gfIL-11, and SignalP was utilized to predict the signal peptide. Multiple sequence alignment was carried out by CLUSTAL-W (http://www.genome.jp/tools-bin/clustalw), and images were generated using ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The p-distance method of the MEGA 11 program, with values expressed as percentages, was employed to perform the gfIL-11 phylogenetic analysis, primed 10,000 times.

Real-time quantitative PCR (qPCR) analysis

Goldfish IL-11 qPCR primers were designed using Primer Express software (Applied Biosystems, USA) considering primer sequences, melting temperature (Tm), GC content, and amplicon length. Tissues and cell populations were meticulously collected from six individual goldfish ($n = 6$), and RNA was extracted using Trizol reagent (Omega Biotech, China) following standardized procedures to ensure integrity. Reverse transcription was performed with attention to detail, using the PerfectStart Green qPCR SuperMix (TransGen, China). Primers were deemed acceptable for use if the experimenetally derived R2 values from the cDNA dilution curves were greater than 0.980. QPCR product was resolved by agarose gel electrophoresis and the resulting product sequenced to confirm primer specificity. Quantitative PCR was executed on the ABI QuantStudio 5 instrument (Thermo Fisher Scientific, USA), with thorough instrument calibration and quality control. The thermocycling protocol, set at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 °C for 1 min, was carefully executed. GraphPad Prism (Applied Biosystems, USA) facilitated rigorous data analysis, including RQ value standardization to gfIL-11 expression in muscle and immune cells. The above processes were performed strictly to ensure that qPCR met the MIQE standard [24].

Isolation of goldfish head kidney leukocytes (HKLs)

HKLs were isolated from goldfish using standard protocols [25]. Briefly, the kidneys were excised and kept in ice-cold NMGFL-15 medium. The tissues were then homogenized, and the cell solution was suspended on top of 51 % Percoll and centrifuged at 400 *g* for 25 min at 4 °C to remove debris. The cells at the 51 % Percoll/medium junction

were collected and washed twice with incomplete medium before resuspending the HKLs in complete medium containg 10 % newborn calf serum, 5 % carp serum, 0.2 % gentamicin, 100 U/mL penicillin, 100 μg/mL strephomycin and 100 μg/mL gentamicin. Viable HKLs were counted using the trypan blue exclusion method before use in experiments.

Analysis of IL-11 expression in activated goldfish HKLs

To prepare heat-killed and alive bacteria, the methods previously described were used [26]. Six goldfish were used for this study ($n = 6$), and leukocytes from their kidneys were utilized to investigate gfIL-11 expression. Leukocytes were grown in a 24-well plate at a concentration of 2×10^6 cells/mL and treated with live *A. hydrophila* (1×10^7 CFU/mL), heat-killed *A. hydrophila* (1×10^7 CFU/mL), or LPS (10 μg/mL, Sigma L2630). Each treatment group consisted of 1×10^6 cells in 500 μL of complete NMGFL-15 medium. After the cells were challenged, they were kept alive at 26°C for 6 h and 12 h. The TranScript Uni All-in-One First-Strand cDNA synthesis Supermix (Transgen, China) was used to synthesize cDNA. The reference gene EF-1α was used to calculate the relative expression levels of gfIL-11.

Prokaryotic expression and scale up production of recombinant goldfish recombinant IL-11

The sequence of gfIL-11 ORF excluding the signal peptide was amplified using specific primers designed to include *EcoR* I and *Hind* III at their 5′ end (Table S1). After PCR amplification, the product was digested with *EcoR* I and *Hind* III restriction enzymes (Thermo Fisher Scientific, USA) and then inserted into the *EcoR* I/*Hind* III-digested pET-32a (+) vector. This resulted in the creation of the recombinant plasmid pET-His-gfIL-11, which was subsequently converted into competent *E. coli* (BL21/DE3) cells (TransGen, China). To express the RgfIL-11 protein, 0.1 mM IPTG induction was carried out at 16 °C overnight, and recombinant protein expression was analyzed by SDS-PAGE. Purification of the recombinant protein was accomplished by using Ni-NTA Sefinose Resin (Sangon, China) as directed by the manufacturer. As a control protein in subsequent experiments, the pET32a (+) vector containing a thioredoxin tag without an insert was expressed and purified in the same manner. Subsequently, a ProteoSpin Endotoxin removal column (Norgen Biotek, USA) was used to purify the recombinant protein after it was dialyzed overnight at 4 °C in 1 x PBS. Micro BCA Protein Assay Kit (Beyotime, China) was used to quantify the protein concentration.

Western blot analysis

The recombinant rgIL-11 and recombinant thioredoxin (rTrx) were analyzed by western blotting. After separation by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and blocked using 5 % (wt/vol) skim milk in TBST buffer (0.05 % Tween-20 in TBS, pH 7.4) at room temperature for 2 h. Subsequently, the membrane was incubated with anti-His-primary antibody (1:3000) overnight at 4 °C and then washed and incubated with a secondary antibody [HRP-conjugated goat anti-mouse (1:5000)] for 1 h at room temperature. Detection of the recombinant proteins was performed using the Tanon 5200 Chemiluminescent Imaging System (Tanon, China).

Detection of cell proliferation of HKLs with MTT assay

Goldfish HKLs suspension (1×10^5 cells/mL) was seeded into 96-well plates and treated with rgIL-11 at different concentrations (0.1, 0.5, 1, and 5 μg/mL) for 24 or 48 h. As a control, rTrx was added at 5 μg/mL. After centrifugation at 230 *g* for 5 min, MTT was added to each well and incubated for an additional 4 h. The formazon salts were dissolved by adding DMSO, and the optical density was measured at 540 nm using a microplate reader (Bio-rad, USA). Three independent experiments were conducted. Cell viability = $(A_{treatment} - A_{blank})/ (A_{control} - A_{blank}) \times 100$ % (where, A = absorbance and DMSO alone as a blank absorbance).

Flow cytometric analysis of goldfish HKLs

The flow cytometric analysis was performed with carefully optimized instrument settings, including appropriate voltage and compensation adjustments. Gating strategies based on forward scatter (FSC) and side scatter (SSC) parameters were employed to identify viable cells and isolate specific leukocyte populations. After isolation, goldfish leukocytes were seeded in a 6-well plate at 1×10^6 cells/well and treated with rgIL-11 at concentrations of 0.1, 0.5, 1, and 5 μg/mL or with rTrx at a final concentration of 5 μg/mL serving as the control. Samples were analyzed using a Fluorescence-activated cell sorter (FACS) Calibur flow cytometer (Becton Dickinson, USA) after 24 h and 48 h of incubation at 20 °C. The analysis was conducted on three different fish ($n = 3$), and involved measuring the forward (size) and side (internal complexity) scatter light patterns of the cells in each treatment group.

Pro-inflammatory cytokine and transcription factor gene expression in goldfish HKLs triggered by rgIL-11

To evaluate the effect of rgIL-11 on goldfish leukocytes, six different fish were used to create primary cultures that were then exposed to 5 μg/mL of rgIL-11 and 5 μg/mL of rTrx (as a control). TRIzol was used to extract RNA from the cells after they had been incubated at 26°C for 6 h and 12 h. Quantitative PCR was used to examine the mRNA levels of a variety of transcription factor genes (MafB, cJun, GATA2, Egr1, cMyb, PU.1, and Runx1) and pro-inflammatory cytokine (IFNγ, IL-1β, and TNF-α) relative to the housekeeping gene EF-1α. In a final volume of 500 μL of complete NMGFL-15 media, 1×10^6 cells were used for each treatment group.

Statistical analysis

All experimental data were analyzed using one-way ANOVA, and then compared to the control and treatment groups using Dunnett's post hoc test $P < 0.05$ was used as the threshold for significance.

Results

Sequence analysis and characterization of goldfish IL-11

The nucleotide sequence of gfIL-11 has been submitted to the Genbank under the accession No. OQ376572. The gfIL-11 cDNA transcript has an open reading frame (ORF) consisting of 591 bp, which encodes 196 aa, comprising a mature peptide of 170 aa and a signal peptide of 26 aa as shown in Supplementary Fig. 1 (Fig. S1). A conservative IL-11 superfamily domain was predicted and observed between positions 31 and 93 aa. The theoretical molecular weight (Mw) and isoelectric point (*p*I) of gfIL-11 are 22.91 kDa and 8.28, respectively. Based on multiple sequence alignment, gfIL-11 has the highest sequence identity with IL-11 homologs from *P. promelas* (92 %), *O. macrolepis* (91 %), *C. carpio* (90 %), and *M. amblycephala* (76 %), and the lowest identity (19–24 %) with those from mammals, reptiles, and aves (Table S2). In addition, the secondary structure of gfIL-11 is similar to that of human IL-11 (Fig. S2). To study the phylogenetic relationships of gfIL-11, homologous amino acid sequences from other teleost fishes and non-fish animals were obtained from NCBI database to construct a phylogenetic tree among different vertebrate species. Thus, a p-distance tree was constructed using MEGA 11 software with the Maximum Composite Likelihood method. Fig. 1 demonstrates that gfIL-11 was categorized with other teleost IL-11, and its evolutionary nearest connection was with *P. promelas* IL-11. This suggests that the phylogenetic tree reflects genetic consistency between these species throughout evolution.

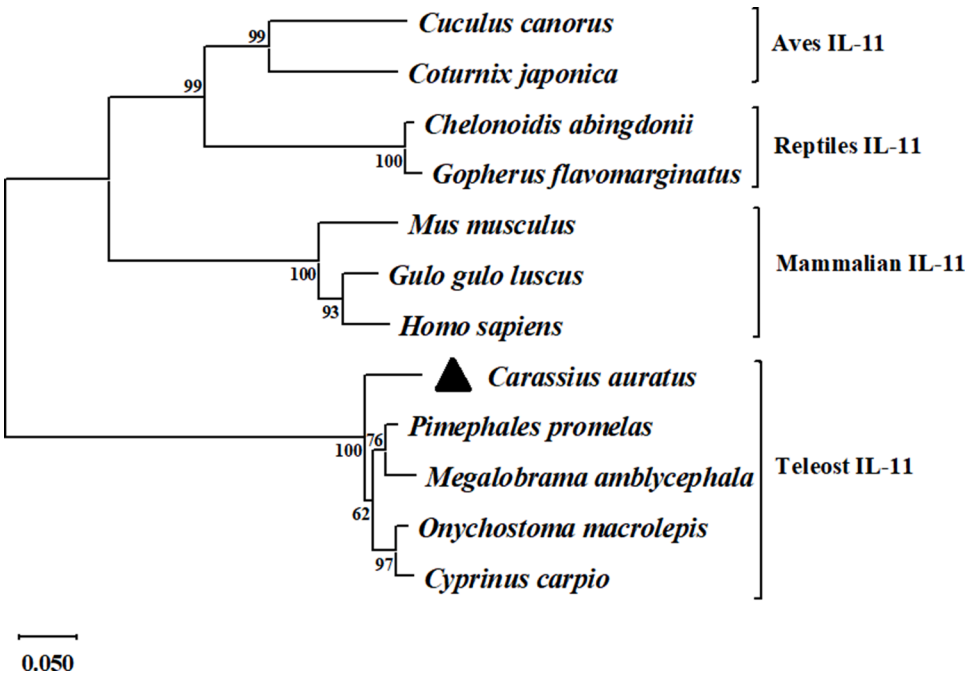

Fig. 1. Phylogenetic analysis of gfIL-11 from different species. The evolutionary history was inferred using the p-distance method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using p-distance method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA 11.

Analysis of IL-11expression in healthy goldfish tissues

qRT-PCR was performed in eight tissues of healthy goldfish using the tissue (muscle) with lowest mRNA expression as a reference tissue. As shown in Fig. 2, the mRNA expression of gfIL-11 was detected in all tested organs. Expression analysis of gfIL-11 indicated that the highest mRNA level of IL-11 was present in the gill, followed by spleen and intestine (Fig. 2). Relatively lower mRNA levels were observed in the heart, liver and muscle (Fig. 2).

Analysis of IL-11, TNF-α and IL-1β expression in A. hydrophila infected goldfish tissues

In teleosts, the kidney serves as a crucial organ for immune function and also plays a role in hematopoiesis and macrophage development [27]. As a first step in investigating the possible inflammatory effects of cytokines in goldfish, we analyzed the expression of IL-11, TNF-α, and IL-1β in both infected and uninfected fish kidneys. The qRT-PCR data

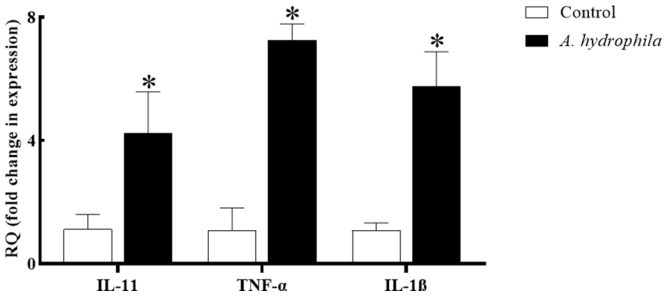

Fig. 3. Expression analysis of IL-11, TNF-α and IL-1β in kidney tissues obtained from day-7 *A. hydrophila* infected goldfish. The expression of IL-11, TNF-α and IL-1β were measured relative to endogenous control gene, elongation factor 1 alpha (EF-1α). Relative tissue expression from six individual fish ($n = 6$), qPCR was done in triplicate for each tissue. All results were normalized against kidney treated by PBS as a control. Significance is denoted by (*) compared to the reference sample ($P < 0.05$).

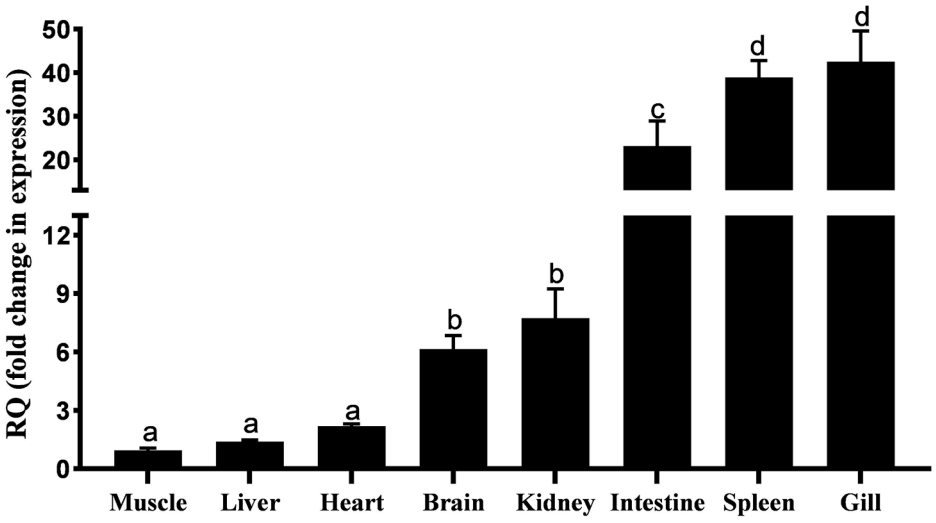

Fig. 2. Expression analysis of IL-11 in tissues obtained from normal goldfish. Analysis of the relative tissue expression was done using tissues from six fish ($n = 6$), qPCR was done in triplicate for each tissue. The expression of IL-11 was relative to endogenous control gene, EF-1α. All results were normalized to lowest expression tissue (muscle). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. Different letters above each bar denote statistically different ($P < 0.05$), and the same letter indicates no statistical differences between groups.

presented in Fig. 3 demonstrated a marked increase in the expression levels of all three cytokines on the 7th day post-infection.

Analysis of goldfish IL-11, TNF-α and IL-1β expression in HKLs treated with chemical stimuli and bacteria

In order to investigate the effects of immune stimuli on goldfish IL-11, TNF-α and IL-1β mRNA expression, we treated goldfish head kidney leukocytes (HKLs) with LPS, and either live or heat-killed

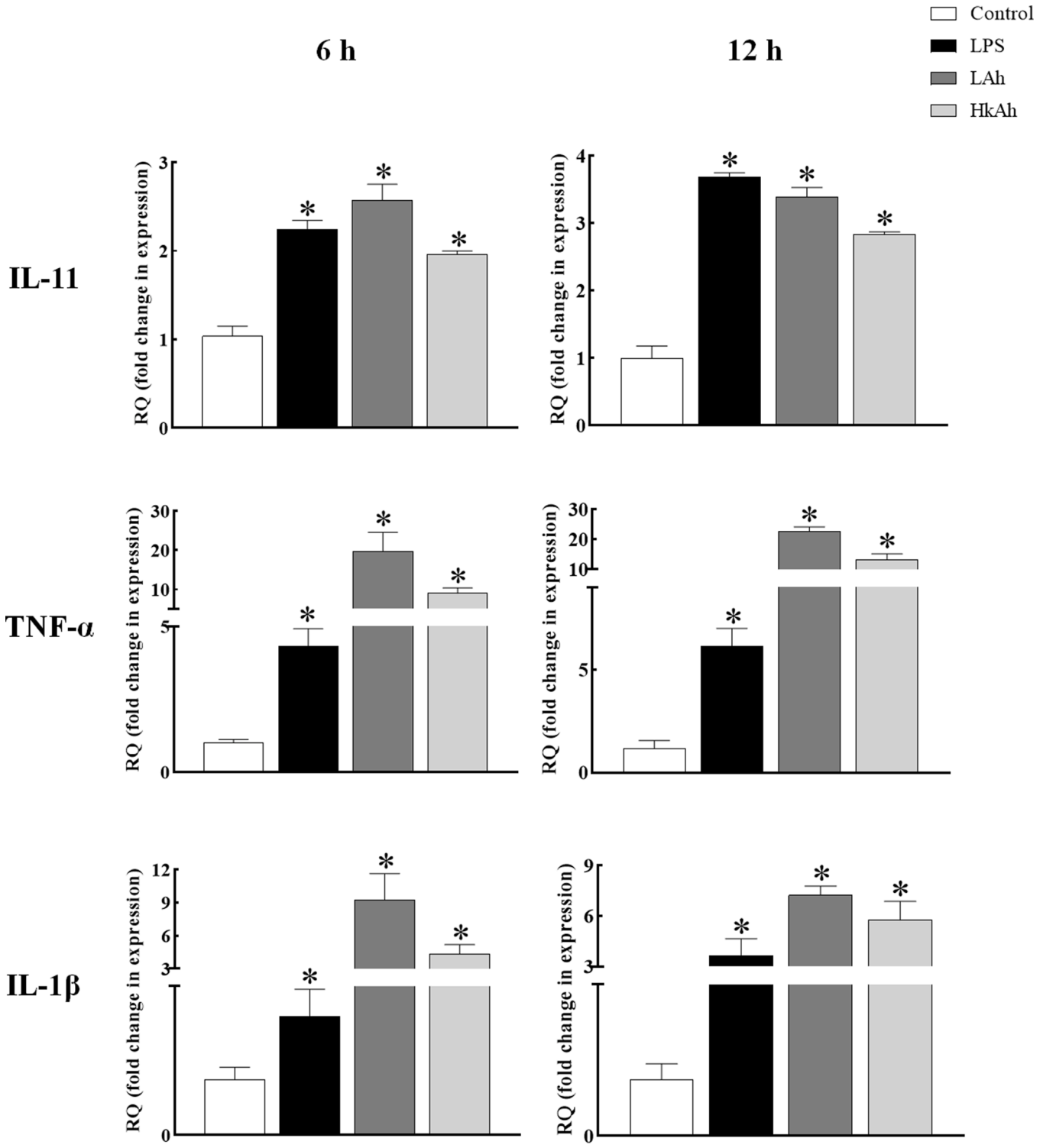

Fig. 4. Quantitative expression of goldfish IL-11, TNF-α and IL-1β in leukocytes treated with different stimuli. The expression of goldfish IL-11, TNF-α and IL-1β in HKLs treated with either rTrx as a control, or lipopolysaccharide (LPS), heat-killed and live *A. hydrophila*. The expression of IL-11, TNF-α and IL-1β were examined relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression values were normalized to the control of each time. Significance is denoted by (*) compared to the reference sample ($P < 0.05$).

A. hydrophila for a period of 6 and 12 h. The mRNA levels of IL-11, TNF-α, and IL-1β were measured in HKLs exposed to LPS, as well as in those exposed to live or heat-killed *A. hydrophila* at 6 and 12 h. The qRT-PCR results showed a significant increase in the expression of all three cytokines after exposure to LPS and *A. hydrophila*, as demonstrated in Fig. 4.

Expression and purification of recombinant IL-11 in goldfish

We aimed to investigate the functional activities of gfIL-11 by expressing the rgIL-11 in *E. coli* and purifying it with the MagneHis protein purification system. SDS-PAGE analysis revealed one band at 43 kDa, consisting of a six-histidine tag and a Trx fusion protein at the N-terminus, as shown by Commassie blue staining (Fig. S3). Western blotting with an anti-His-antibody further verified the recombinant (Fig. S3).

Effects of rgIL-11 treatment on cell proliferation of goldfish HKLs

To examine whether rgIL-11 could induce the proliferation of immune cells, cultured HKLs were incubated with either rTrx at a final concentration of 5 μg/mL as control or rgIL-11 at the concentrations of 0.1, 0.5, 1, and 5 μg/mL, respectively. MTT assay was performed, and the results indicated that treatment with different concentrations of rgIL-11 induced a significant proliferative response in cultured HKLs compared to the control group after 48 h (Fig. 5).

Flow cytometric results of goldfish HKLs

To assess the impact of rgIL-11 on cell differentiation, we treated sorted goldfish leukocytes with varying concentrations of the cytokine (0.1, 0.5, 1, and 5 μg/mL) and monitored them for 48 h using flow cytometry. The findings showed that rgIL-11 treatment at 1 μg/mL had the greatest effect on differentiation in sorted leukocyte subpopulations, as presented in Fig. 6.

Proinflammatory cytokine analysis in rgIL-11-treated goldfish HKLs

We investigated the effect of rgIL-11 on the mRNA expression levels of IFNγ, IL-1β, and TNF-α genes in goldfish HKLs cultures (Fig. 7). Treatment with 5 μg/mL rgIL-11 resulted in significant upregulation of TNF-α mRNA levels at 6 h and IFNγ at 6 h and 12 h, while there was no significant effect on IL-1β mRNA production (Fig. 7).

Transcription factor analysis in rgIL-11-treated goldfish HKLs

To investigate the potential involvement of growth factor IL-11 in the modulation of transcriptional factors, we treated HKLs with 5 μg/mL rgIL-11 and assessed the mRNA levels of MafB, cJun, GATA2, Egr1, cMyb, PU.1, and Runx1 at 6 and 12 h. We found a significant upregulation of MafB, cJun, GATA2, and Egr1 mRNA levels at 6 h (Fig. 8A), and of MafB, cJun, and GATA2 mRNA levels at 12 h post-treatment (Fig. 8B). However, cMyb, PU.1, and Runx1 mRNA levels were not shown to be significantly different across any time point (Fig. 8).

Discussion

IL-11 is an important cytokine in the control of hematopoietic progenitor differentiation and proliferation. It also plays a role in the defense of mucosal epithelia, adipogenesis, and bone formation [15]. Despite its significant contribution to a variety of physiological functions, the hematopoietic properties of many growth factors have not yet been well studied in fish, including IL-11. This paper reports on the identification and functional characterization of a goldfish IL-11 molecule, indicating its potential role in the proliferation, differentiation, and immune response of goldfish HKLs.

The goldfish IL-11 gene was originally cloned and isolated from our previously generated transcriptome database [23]. The ORF of the gfIL-11 cDNA transcripts consisted of 591 bp with its ORF encoding 196 aa, which is almost similar to other teleost fish species [6,19] and human [8], indicating conserved structural features of IL-11 in vertebrates throughout evolution. The phylogenetic tree showed that gfIL-11 was grouped with IL-11 of other teleosts. Similar to previous studies [6,19], gfIL-11 was grouped with fish all belonging to the class Actinopterygii, in particular with *P. promelas,* suggesting that IL-11 s in teleosts have been evolutionarily conserved. Moreover, although this gene showed a close relationship with other fish species, the phylogenetic tree presents us with a certain gap with mammals. Thus, it is essential to clarify the relationship between teleosts and mammals in terms of different functionality regarding IL-11 gene. Furthermore, expression analysis indicated that gfIL-11 had relatively high levels in gills, spleen, intestine, and the lowest mRNA level was observed in heart, liver and muscle. These results were consistent with previous studies on the tissues distribution of IL-11 in *T. ovatus* [18], *O. mykiss* [6], *P. olivaceus* [12], *C. carpio* [15], and L. *crocea* [19].

Although IL-11 is a commonly used drug and has various essential functions in the immune system of mammals [10], the role of IL-11 and its receptors in the immune responses of fish species have not been extensively studied. This research examined expression of IL-11 in

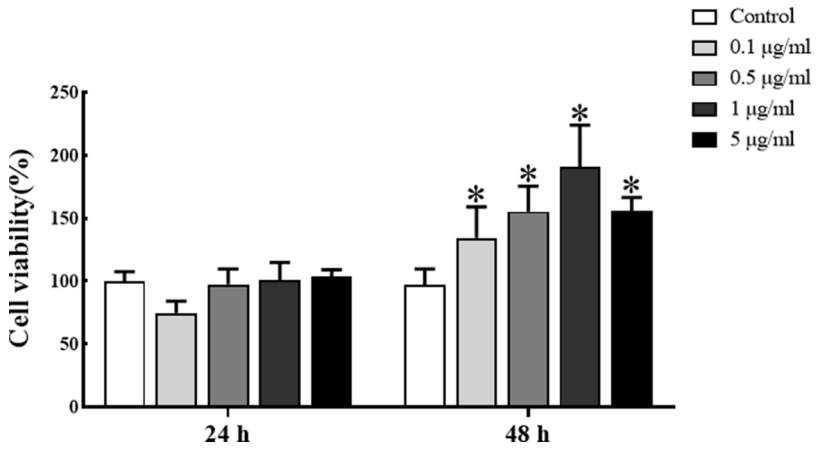

Fig. 5. Effects of rgIL-11 on the viability of goldfish leucocytes using MTT assay. Goldfish head kidney leucocytes were treated with or without rgIL-11 at different concentrations for 24 h and 48 h. The proliferation of the cells was determined by MTT assay. Data are presented as means ± SEM ($n = 3$). Significance is denoted by (*) compared to the reference sample ($P < 0.05$).

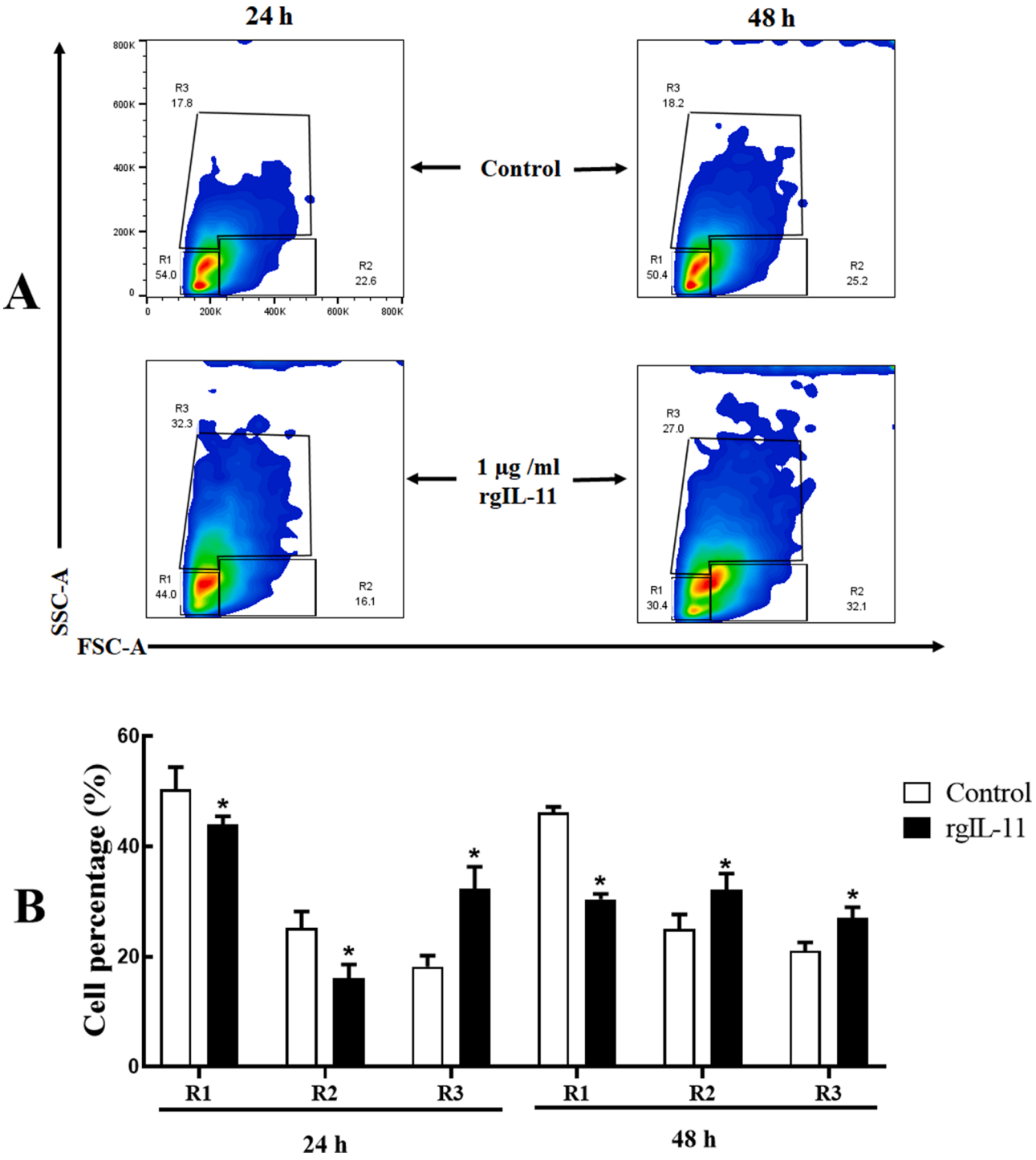

Fig. 6. Flow cytometric analysis showing the differentiation of goldfish progenitors, after treatment with 1 μg/mL rgIL-11. (A) Analysis shows the results obtained at 24 h and 48 h taken from three individual fish. The gates (R1-R3) determine a chronologic sequence of cell development. Leukocyte differentiation progresses through R1, R2, and R3 after treatment. (B) Bar graph shows the percentage of cells in R1, R2, and R3 after treatment with 1 μg/mL rTrx as a control and of rgIL-11 for 24 h and 48 h. Data are presented as means ± SEM ($n = 3$). Significance is denoted by (*) compared to the reference sample ($P < 0.05$).

goldfish kidneys infected with *A. hydrophila* to determine its role in inflammation in fish. On day seven following infection, gfIL-11 expression was considerably elevated by qRT-PCR. Pro-inflammatory cytokine mRNA levels were found to be elevated on day 7 in previous studies [25, 28] indicate that gfIL-11 may potentially play a pro-inflammatory role in response to *A. hydrophila* infection in fish.

To investigate whether the *A. hydrophila* can activate IL-11 in goldfish, we exposed primary kidney-derived HKLs to live or heat-killed *A. hydrophila* for either 6 or 12 h. Our results showed that LPS stimulation significantly increased the mRNA level of gfIL-11 at 6 h and 12 h, with the peak occurring at 12 h when compared to the time-matched controls. Furthermore, heat-killed and live *A. hydrophila* were able to induce significantly gfIL-11 mRNA production from 6 h to 12 h. The observed up-regulation of IL-11 transcripts in our study conforms with prior reports of increased IL-11 mRNA expression in response to LPS and bacterial challenge in rainbow trout [6] and in immunological tissues or immune cells of other fish species such as common carp [15], Japanese flounder [12], crucian carp [29], golden pompano [18], cobia

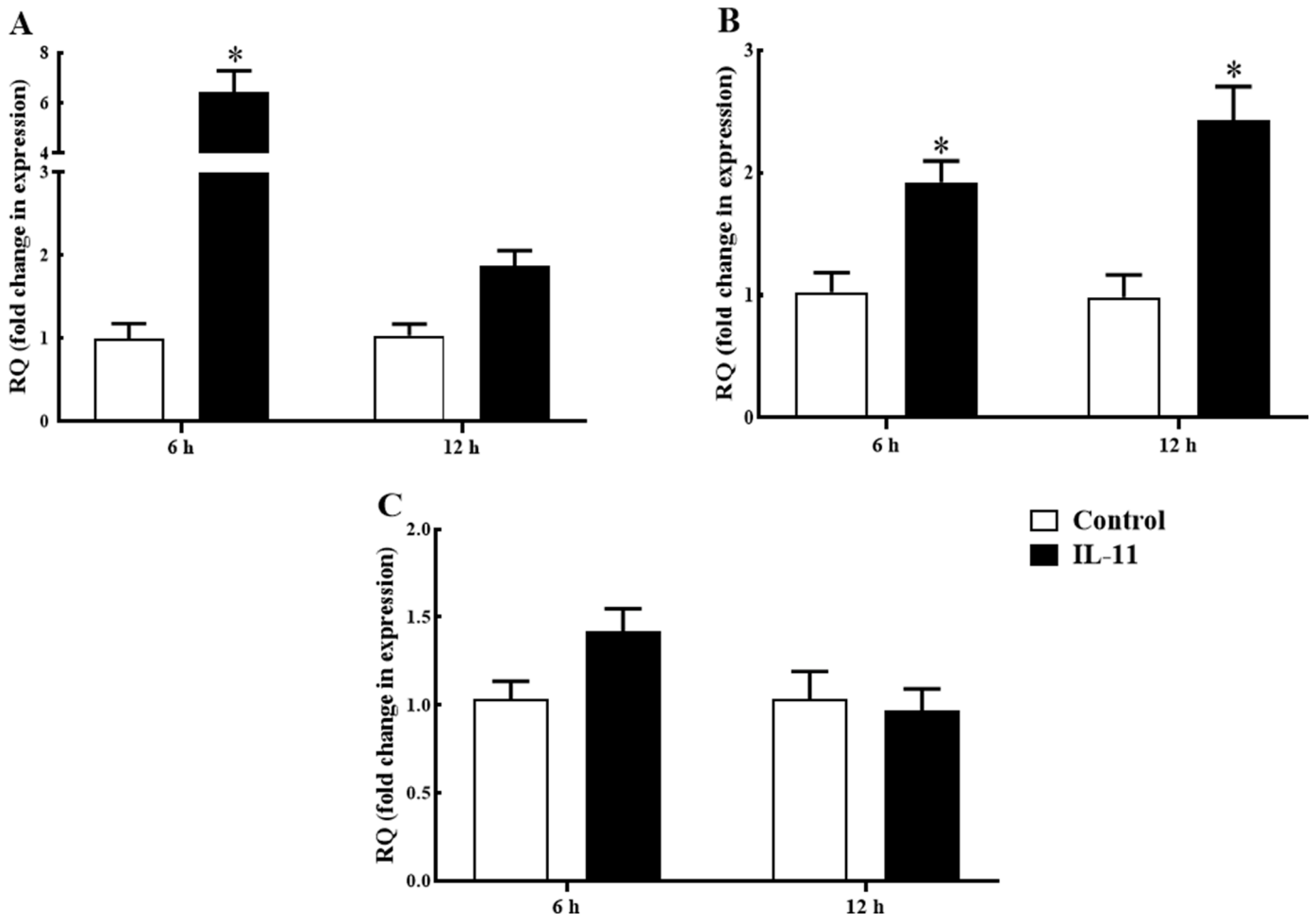

Fig. 7. Quantitative expression analysis of goldfish proinflammatory cytokines (TNF-α, IL-1β and IFNγ) in HKLs treated with 5 μg/mL rgIL-11 for 6 h and 12 h. The expression of goldfish IL-11 was examined against the endogenous control gene, elongation factor 1 alpha (EF-1α). Expression values were normalized to those seen in rTrx -treated cells. Results are the mean ± S.E.M. of primary leukocyte cultures established from six individual fish ($n = 6$).

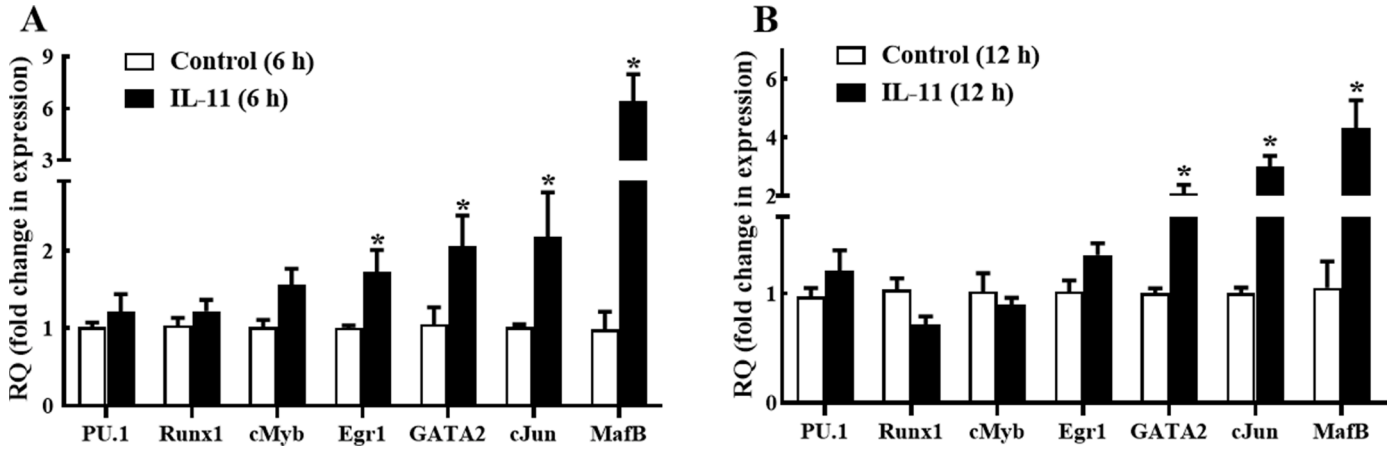

Fig. 8. Quantitative expression analysis of goldfish transcriptions factors in HKLs treated with 5 μg/mL rgIL-11 for 6 h (A) and 12 h (B). The expression of goldfish IL-11 was examined against the endogenous control gene, elongation factor 1 alpha (EF-1α). Expression values were normalized to those seen in rTrx -treated cells. Results are the mean ± S.E.M. of primary leukocyte cultures established from six individual fish ($n = 6$).

[17], and large yellow croaker [19]. Moreover, IL-11 mRNA up-regulation has also been noticed in humans after LPS treatment [30], and in mice after infection with *Pseudomonas aeruginosa* [31]. Our study confirms previous findings that indicate upregulation of IL-11 expression in fish in response to bacterial challenge. Our results show that HKLs from goldfish treated with LPS and live *A. hydrophila* exhibit significantly increased gfIL-11 expression, suggesting a potential role for IL-11 in fish immune defense against bacterial and viral infections, similar to that observed in mammals.

In mammals, IL-11 has been shown to promote the activation of cell

survival and proliferation, stimulate hemopoiesis and thrombopoiesis, regulate macrophage differentiation, proliferate "IL-6-dependent" plasmacytoma cells, confer mucosal protection and accelerate megakaryocytopoiesis [5,9,10,11]. Additionally, a greater number of both early and late hematopoietic progenitors proliferate and differentiate in response to IL-11., either alone or in combination with other growth factors [7,32]. To examine whether rgIL-11 could induce the proliferation of immune-related cells, cultured HKLs were incubated with rTrx as control or rgIL-11 at different concentrations. MTT assay was conducted, and the results showed after 48 h, treatment with rgIL-11 induced a proliferative response at all the examined concentrations, with significant proliferation observed in cells treated with 1 μg/mL of rgIL-11. Moreover, rgIL-11 at various concentrations was also added to sorted goldfish leukocyte cells. Leukocyte subpopulations were monitored every 24 h over a 2-day period via flow cytometry to assess the effects of rgIL-11 on their differentiation. Our results indicate that at a concentration of 1 μg/mL, rgIL-11 induced differentiation in the sorted leukocytes. These observations suggest that rgIL-11 treatment of leukocyte subpopulations can promote proliferation and differentiation of leukocytes into macrophages, highlighting the functional importance of this molecule in myelopoiesis.

Furthermore, IL-11 has been found to have immunomodulatory properties in mammalian models [33,34]. In this study, we investigated the effect of gfIL-11 on pro-inflammatory cytokine production by treating goldfish HKLs with rgIL-11 and assessing mRNA and protein levels of IFNγ, IL-1β, and TNF-α. Treatment with rgIL-11 resulted in significantly elevated expression of TNF-α at 6 h and IFNγ at 6 h and 12 h. However, the expression levels of IL-1β did not significantly change after rgIL-11 treatment. Our results are consistent with a previous study showing that IL-11 from large yellow croaker significantly enhanced TNF-α expression levels but not IL-1β [19]. Therefore, fish IL-11 may play an important role in pro-inflammatory responses by activating TNF-α and IFNγ.

To improve our knowledge of hematopoietic cells in teleosts in general and goldfish in particular, mRNA levels of transcription factors were utilized as a tool to investigate how gfIL-11 influences the differentiation of progenitor cell populations. Thus, treatment of HKLs with rgIL-11 showed significant upregulation of mRNA levels of the myeloid transcription factor MafB, cJun, GATA2, and Egr1, all known to be involved in monocytes/macrophages cell lines [35]. As we known, MafB has been reported to stimulate the differentiation of myeloblasts into monocytes and macrophages [36–39], and to have a high level of expression in monocytes/macrophages [35]. The function of MafB in teleost myelopoiesis remains largely unknown. However, a study using the goldfish model detected a MafB transcript and observed an increase in MafB mRNA during macrophage development in the primary renal macrophage culture system [40]. Myeloid progenitors can be differentiated into monocytes or macrophages, and cJun has been demonstrated to play a role in this process in previous research [35,41]. Moreover, GATA2 is a transcription factor that is involved in myeloid gene activation as well as the maintenance and proliferation of multipotent progenitor cells [35]. As for Egr1, it has been shown to be essential to differentiate myeloblasts into macrophages or monocytes and favors macrophage lineage at the expense of granulocytic lineage [42]. These results reinforce our understanding that rgIL-11 is strongly involved in cell proliferation and differentiation of myeloid lineage.

In summary, we provided insights into the functionality of gfIL-11 by showing that rgIL-11 not only stimulated the proliferation and differentiation of primary head kidney cells, but also upregulated the expression of proinflammatory cytokines in HKLs including TNF-α and IFNγ. The treatment with rgIL-11 resulted in a significant increase in the expression of key transcriptional regulators, such as MafB, cJun, GATA2, and Egr1. The results of this study suggest that gfIL-11 may play a crucial role in regulating the proliferation, differentiation, and immune response of goldfish progenitor cells. In contrast to prior investigations that have extensively elucidated the immunological function of IL-11 in fish, the present study stands out by offering valuable insights into its significance within the hematopoietic process. Thus, our data provided the fundamental basis for the regulation of IL-11 in inflammatory response and hematopoiesis in teleost.

CRediT authorship contribution statement

Moussa Gouife: Data curation, Investigation, Methodology, Writing – original draft. **Songwei Zhu:** Investigation, Methodology. **Kejing Huang:** Investigation, Methodology. **Mateen Nawaz:** Investigation, Methodology. **Xinyuan Yue:** Investigation, Methodology. **Rongrong Ma:** Project administration, Resources. **Jianhu Jiang:** Project administration, Resources. **Suming Zhou:** Project administration, Resources. **Jiasong Xie:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2023.100117.

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