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Molecular cloning, characterization and gene expression analysis of twelve interleukins in obscure puffer *Takifugu obscurus*

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ABSTRACT

Interleukins (ILs) are a subgroup of secreted cytokines, which are molecules involved in the intercellular regulation of the immune system. In this study, 12 IL homologs were cloned and functionally identified from obscure puffer *Takifugu obscurus*, and they were termed as *ToIL-1β, ToIL-1, ToIL-6, ToIL-10, ToIL-11, ToIL-12, ToIL-17, ToIL-18, ToIL-20, ToIL-24, ToIL-27*, and *ToIL-34*. Multiple alignment results showed that except for ToIL-24 and ToIL-27, other deduced ToIL proteins shared typical characteristics and structure with other known fish ILs. Phylogenetic analysis revealed that 12 ToILs were evolutionarily closely related to their counterparts in other selected vertebrates. Tissue distribution assay demonstrated that the mRNA transcripts of most *ToIL* genes were constitutively expressed in all tissues examined, with relatively high expression in immune tissues. Following *Vibrio harveyi* and *Staphylococcus aureus* infection, the expression levels of 12 *ToILs* in the spleen and liver were significantly upregulated, and their response over time varied. Taken together, these data were discussed accordingly with the *ToIL* expression and the immune response under the different situations tested. The results suggest that the 12 *ToIL* genes are involved in the antibacterial immune response in *T. obscurus*.

1. Introduction

Cytokines are small glycoproteins that act as major regulators of innate and adaptive immunity that enable cells of the immune system to communicate over short distances [1]. Interleukins (ILs) are a subgroup of secreted cytokines, which are molecules involved in the intercellular regulation of the immune system. The term interleukin was first coined in 1979 to refer to molecules that signal between different leucocyte types, although this communication is not exclusively restricted to leucocyte communication [2]. To date, dozens of ILs are produced by various cell types, and many of them are synthesized by immune cells such as T lymphocytes, B lymphocytes, and macrophages/monocytes cells [3,4]. Most ILs are conserved from fish to mammals and play essential roles in physiological inflammatory and immunological processes [5,6].

Fish comprises the first group in the animal evolution that possess both innate and adaptive immunity [7]. In the past two decades, considerable progress has been made in the cloning, sequencing, and functional analysis of fish IL genes [5]. In fish, the first IL-1β cDNA was identified from rainbow trout *Oncorhynchus mykiss* by cDNA library screening [8]. Subsequently, IL-1βs have been found in several teleost and cartilaginous fish species, such as catshark *Scyliorhinus canicula* [9], channel catfish *Ictalurus punctatus* [10], and Nile tilapia *Oreochromis niloticus* [11], and fish IL-1β proteins are involved in regulating the inflammatory response to bacterial or parasitic infection [12]. In comparison with IL-1β, fish IL-18 s are under-investigated and have been reported only in a few fish species, including rainbow trout [13], seabream *Sparus aurata* [14], turbot *Scophthalmus maximus* [15], and elephant shark *Callorhinchus milii* [16]. Studies have suggested that IL-18 may play a role in mucosal immunity [14]. In teleosts such as Japanese pufferfish *Fugu rubripes* [17], gilthead seabream *S. aurata* [18], zebrafish *Danio rerio* [19], and Siberian sturgeon *Acipenser baeri* [20], IL-6 s play vital roles in inflammatory response, driving lymphocyte differentiation and the induction of antimicrobial peptides. IL-10 s have been identified in model fish, such as zebrafish *D. rerio* [21], and economically important species, such as grass carp *Ctenopharyngodon*

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idella [22], common carp *Cyprinus carpio* [23], and golden pompano *Trachinotus ovatus* [24]. IL-10 in common carp exerts prototypical anti-inflammatory activities on macrophages and neutrophils; it down regulates oxygen and nitrogen radical production and cytokine synthesis by inhibiting pro-inflammatory gene expression [23]. IL-17 family genes play crucial roles in fish immune responses by stimulating the release of immune factors in different ways. In large yellow croaker *Larimichthys crocea*, IL-17C can strongly stimulate the expression of chemokines, pro-inflammatory factors, and antibacterial peptide and may promote the inflammatory response and host defense by activating the NF-κB signaling [25]. The members of IL-20 subfamily have been described in green pufferfish *Tetraodon nigroviridis*, zebrafish, and rainbow trout and play important roles in homeostasis, cell proliferation, and host immune defenses [26]. IL-34 in rainbow trout [27], large yellow croaker [28], and grouper *Epinephelus coioides* [29] responds to stimulation by pathogens and certain pathogen-associated molecular pattern molecules. Grass carp IL-34 can induce the expression of pro-inflammatory cytokines in primary head kidney macrophages [30]. The increasing knowledge based on the regulation of immune responses in fish by ILs and their potential use as good immunopotentiator candidate for disease prevention or treatment in fish make fish IL biology an attractive and rapidly expanding field.

Takifugu obscurus, commonly known as obscure puffer, is an anadromous species that migrates to freshwater from the sea for spawning [31]. In recent years, the immune system of *T. obscurus* has attracted widespread attention because of its important evolutionary status and great economic value. *T. obscurus* is among the useful models for studying the immune system of fish. Thus, the systematic study of different types of cytokines in *T. obscurus* is expected to lay the foundation for in-depth studies of the regulatory mechanism of fish immunity. In the present study, the structural and functional features of ILs in *T. obscurus* were investigated. Based on the data from a previously constructed transcriptome, 12 *IL* genes were identified from *T. obscurus*, namely, *ToIL-1β, ToIL-1, ToIL-6, ToIL-10, ToIL-11, ToIL-12, ToIL-17, ToIL-18, ToIL-20, ToIL-24, ToIL-27*, and *ToIL-34*. The sequence alignment, phylogenetic relationship, and mRNA tissue distribution of the 12 *T. obscurus* ILs were comparatively analyzed. Furthermore, the inducible expression of these *ToIL* genes was detected in different tissues following exposure to *Staphylococcus* and *Vibrio*. Overall, this study may provide evidence for the 12 *ToIL* genes in *T. obscurus* with characterization of their gene expression and function.

2. Materials and methods

2.1. Experimental fish

Healthy *T. obscurus* (approximately 50 g in weight) were obtained from a breeding farm in Yangzhong City, Jiangsu Province, China. All fish samples brought to the laboratory were used during the assay. They were cultured in a recirculating aquaculture system at 23–25 °C for 2 weeks until the start of the experiment. During this period, the fish samples were kept under standard rearing conditions and fed with commercial pellets regularly.

2.2. Bacterial challenge and sample collection

Staphylococcus aureus ATCC 25,923 was purchased from China Center of Industrial Culture Collection. *Vibrio harveyi* E385 was kept in our laboratory. The tissue distribution of 12 *ToIL* genes was investigated by obtaining three samples of heart, liver, kidney, spleen, gills, and intestine tissues from normal *T. obscurus*. Each tissue sample was pooled from three individual fish. For immune challenge, fish samples were intraperitoneally injected with 100 μL of *S. aureus* (3.0×10^7 CFU/mL) and *V. harveyi* (3.0×10^7 CFU/mL). The spleen and liver were sampled. Three samples of spleen and liver (each pooled from three fish) were sampled at 0, 12, 24, 36, 48 and 72 h post-injection in each group. Fish injected with phosphate-buffered saline (PBS,140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) were sampled as controls.

2.3. RNA extraction and cDNA synthesis

From all samples, total RNA was extracted using the TRIzol reagent kit (Invitrogen, USA) in accordance with the manufacturer's instructions. The extracted RNA was treated with RNase-Free DNase (Takara, Japan) to remove contaminating DNA. RNA concentration was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and RNA integrity was confirmed by 1.5% agarose gel electrophoresis. Total RNA (1 μg) was reverse-transcribed into first-strand cDNA by using the PrimeScript™ II 1st strand cDNA synthesis kit (Takara, Japan) following the manufacturer's instructions. The resulting cDNA was diluted six times and used as template for gene cloning.

2.4. Cloning of T. obscurus ILs

Transcriptome data from *T. obscurus* liver (not shown) were previously obtained in our laboratory. These data were searched for IL homologs by using *Takifugu rubripes* IL sequences as the query by local blast. Twelve pairs of primers were designed to amplify the complete open reading frames (ORFs) of 12 *ToIL* genes (*ToIL-1β*-F and *ToIL-1β*-R, *ToIL-1*-F and *ToIL-1*-R, *ToIL-6*-F and *ToIL-6*-R, *ToIL-10*-F and *ToIL-10*-R, *ToIL-11*-F and *ToIL-11*-R, *ToIL-12*-F and *ToIL-12*-R, *ToIL-17*-F and *ToIL-17*-R, *ToIL-18*-F and *ToIL-18*-R, *ToIL-20*-F and *ToIL-20*-R, *ToIL-24*-F and *ToIL-24*-R, *ToIL-27*-F and *ToIL-27*-R, and *ToIL-34*-F and *ToIL-34*-R, Table 1). PCR amplification was carried out in a 20 μL reaction mix containing 2 μL of 10 × Ex Taq buffer, 1.6 μL of dNTP mix (2.50 mM), 0.4 μL of each primer (10 mM), 0.2 μL of Ex Taq polymerase (Takara, Japan), 1 μL of template cDNA, and 14.4 μL of nuclease-free water. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 30 times of main amplification cycle: 30 s of denaturation at 95 °C, 30 s of annealing at 56 °C and 2 min of extension at 72 °C; and 5 min of final extension at 72 °C. The PCR products were electrophoresed on 1.2% agarose gel to determine successful amplification. The PCR amplified fragments were purified using a DNA gel extraction kit (Generay, China), then cloned into the pEASY-T3 vector (TransGen, China), and transformed into *Escherichia coli* DH5α (TransGen Biotech, China). The positive clones were picked and confirmed by sequencing (Sangon Biotech, China).

2.5. Bioinformatic analysis

The ORF finder program (https://www.ncbi.nlm.nih.gov/orffinder/) and ExPASy (http://web.expasy.org/translate/) were used to deduce the amino acid sequences of ToILs. The BLASTX and BLASTP programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyze the nucleotide and deduced protein sequences, respectively. Conserved domain footprints were analyzed using Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (http://smart.embl-heidelberg.de/). Three-dimensional (3D) models were constructed using the SWISS-MODEL (https://swissmodel.expasy.org/) and PyMOL program. The theoretical isoelectric points (pI) and molecular weight (Mw) of ToILs were predicted using the ExPASy compute pI/Mw tool (http://web.expasy.org/compute_pi/). The Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) and GENEDOC software were used to create multiple sequence alignment by using the full ORF sequences of ToILs. Phylogenetic tree based on Neighbor-Joining (NJ) method was constructed using MEGA 7.0 software [32], with 1000 bootstrap samples.

2.6. Gene expression analysis by RT-qPCR

After tissue collection, total RNA extraction, and cDNA synthesis, gene expression analysis via quantitative real-time PCR (RT-qPCR) was

Table 1
Sequences of the primers used in the study.

Primers name	Sequences (5′–3′)	Tm (°C)
ToIL-1β-F	ATGGAATCTCAGATGAAATCCAACG	63.6
ToIL-1β-R	TTACATCTCTCCCTCACACGTGGGCG	72.1
ToIL-1-F	ATGGACCTGAAGGAATCAGAGGTA	61.1
ToIL-1-R	TCATTTCTTAATGACAAAGAGGAAGG	60.8
ToIL-6-F	ATGGCTTCCATCAGTTACCTGCTCGC	70.7
ToIL-6-R	TCACGATCTCTTCTTTGGGTGGTAG	63.8
ToIL-10-F	ATGACTCCCGGCTCTCTGCTCTCCG	72.8
ToIL-10-R	TCAGGCCACGTTGCGGCGCCTCTTT	79.3
ToIL-11-F	ATGAAATTGCTCCTCGACTCCTCCTC	67.5
ToIL-11-R	TCATTGCACCGCGGCATTGACCTTGG	78.5
ToIL-12-F	ATGGCCTCGCTCCACCTCTGTGAGTA	71.2
ToIL-12-R	TCAGCTGCTGGGCTGAGCATCGGCAC	78.6
ToIL-17-F	ATGCAGGTGGTGTCGGGAACGCTGCT	77.0
ToIL-17-R	GGCCCACTGTTATACCCCAACAGTGA	69.2
ToIL-18-F	ATGGCAGCTAACAATGGCAATTTTG	66.6
ToIL-18-R	CTAAGACATGGTGACGTAGCAGGTC	62.2
ToIL-20-F	ATGAAGACTCTGTCATCCCTCTGCCT	65.9
ToIL-20-R	TCAGTTCGGACTTTTCTGCTCGGAG	68.1
ToIL-24-F	ATGCTCGCCTCCACACTCCGCTGCTC	77.0
ToIL-24-R	TCAGGCCACGGTAACGTTGGTCTGG	72.8
ToIL-27-F	ATGATTGGGTGGACCACTCTGGCTCT	70.1
ToIL-27-R	CTACAGATTGCTTTTGGGTAAAGTGAT	62.1
ToIL-34-F	ATGTGCACGCCCCTGAAGACCATCAA	74.1
ToIL-34-R	TCACACACGTAAGGTGGGCGCCGCGT	79.8
ToIL-1β-qF	ATGAGCATGCAGGAGTACAG	53.1
ToIL-1β-qR	GGTTCACTTTGCGACATTCC	57.8
ToIL-1-qF	AACACGTGGCCTCTCATTAC	54.9
ToIL-1-qR	CTCCTCCTCATTTGCCTTGTAG	58.5
ToIL-6-qF	CGACTTTCTGGACCGTTACAA	57.9
ToIL-6-qR	CCGCTTGAGAAGAACTGAGTAG	56.5
ToIL-10-qF	TTCTACCTGGACTCGGTTCT	53.7
ToIL-10-qR	TGGTCGAAGATCTGCTGAATG	58.5
ToIL-11-qF	GTCGGCCCATAGTCTCAATAATC	59.8
ToIL-11-qR	CCTGTTCAGCCACTCAAAGT	54.9
ToIL-12-qF	GAGGTGGAAGAAGAGAAGGTTG	57.4
ToIL-12-qR	ACAGCTCTGTTGATGGTGATG	55.4
ToIL-17-qF	TACTATCCTACGCTGGTCCTC	54.0
ToIL-17-qR	GTCCTCAGCTGGAACTCATATT	55.4
ToIL-18-qF	GAGAATCACAGAAGGGTGCTATC	57.5
ToIL-18-qR	GGATGACTTTGTTCAGCGTTTG	59.9
ToIL-20-qF	ACGTAGAGAGAGTGTTCAGGAG	52.9
ToIL-20-qR	CGCAGAGGCAGTGACATTTA	57.1
ToIL-24-qF	GCCCAACATCAAGCCAAATC	60.2
ToIL-24-qR	CAGCACGTTGTTCAGGTAGA	54.2
ToIL-27-qF	TGCTGGTGCGTCAGTTATC	54.7
ToIL-27-qR	CAGACTCTGTGTGTCTCTCTCT	50.2
ToIL-34-qF	TATCCAGGAGACTTGGGATCA	56.8
ToIL-34-qR	ATGGTGTAGCACAGGTTGTC	52.6
β-actin-qF	GCATTGTCACCAACTGGGATG	60.8
β-actin-qR	GCAGGACTGGATGCTCCTCT	58.9

carried out using the SYBR Premix Ex Taq™ II kit (Perfect Real Time, Takara, Japan) on LightCycler® 96 (Roche, USA). The amplification reaction was performed using a MicroAmp Optical 96-well reaction plate (Applied Biosystems, USA), with a total reaction volume of 10 μL, consisting of 5 μL of 2 × SYBR Premix Ex Taq™ II, 1 μL of the cDNA template, 0.4 μL each of the sense and reverse primers (10 μM), and 3.2 μL of ddH_2O. The cycling parameters were as follows: 95 °C for 60 s, 95 °C for 5 s, 60 °C for 30 s, then go to step 2 and repeated for 40 cycles. After each RT-qPCR reaction, dissociation analysis was performed to confirm the detection of only one product. *β-actin* was amplified as the reference gene. All primers (*ToIL-1β*-qF and *ToIL-1β*-qR, *ToIL-1*-qF and *ToIL-1*-qR, *ToIL-6*-qF and *ToIL-6*-qR, *ToIL-10*-qF and *ToIL-10*-qR, *ToIL-11*-qF and *ToIL-11*-qR, *ToIL-12*-qF and *ToIL-12*-qR, *ToIL-17*-qF and *ToIL-17*-qR, *ToIL-18*-qF and *ToIL-18*-qR, *ToIL-20*-qF and *ToIL-20*-qR, *ToIL-24*-qF and *ToIL-24*-qR, *ToIL-27*-qF and *ToIL-27*-qR, *ToIL-34*-qF and *ToIL-34*-qR, and *β-actin*-qF and *β-actin*-qR) used in this study were designed using IDT's PrimerQuest™ tool (https://sg.idtdna.com/PrimerQuest/Home/Index) and are listed in Table 1.

2.7. Statistical analysis

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [33] and then normalized using the reference gene *β-actin*. Gene expression data were log-transformed (log base 2) before being subjected to one-way ANOVA. All analyses were implemented in GraphPad Prism v5 (San Diego, CA, USA). The quantitative expression data are presented as the means ± standard deviation (SD, $N = 3$). Statistical significance was determined using Student's *t*-tests. Significance was defined at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Result

3.1. Sequence information of ToIL genes

A total of 12 *ToIL* genes were identified from *T. obscurus* (Supplementary Fig. S1). Based on the results of BLASTP and sequence analysis, these 12 *ToIL* genes were named *ToIL-1β, ToIL-1, ToIL-6, ToIL-10, ToIL-11, ToIL-12, ToIL-17, ToIL-18, ToIL-20, ToIL-24, ToIL-27*, and *ToIL-34*. The complete ORF sequences of these *ToIL* genes had lengths of 771, 1050, 684, 684, 606, 633, 477, 570, 531, 549, 759, and 786 bp, respectively. Twelve *ToIL* genes encoded proteins of 256, 349, 227, 227, 201, 210, 158, 189, 176, 182, 252, and 261 amino acids. The sequences of ToILs were submitted to NCBI GenBank. Detailed information is summarized in Table 2.

Based on functional domain prediction analysis by using the SMART program, the predicted ToIL proteins contained different domains (Fig. 1). ToIL-1β contained an IL1_propep domain (position 1–76) and an IL1 domain (position 92–244). ToIL-1 contained two low-complexity regions (positions 107–129 and 140–147) and an IL1 domain (position 199–346). ToIL-6 contained a signal peptide (position 1–22), a low-complexity region (position 29–46), and an IL6 domain (position 82–203). ToIL-10 contained a signal peptide (position 1–21) and an IL10 domain (position 81–219). ToIL-11 contained a low-complexity region (position 3–17) and an IL11 domain (position 21–196). ToIL-12 contained a signal peptide (position 1–38) and a IL12 domain (position 94–203). ToIL-17 contained a signal peptide (position 1–19) and an IL17 domain (position 59–150). ToIL-18 contained an IL1 domain (position 64–184). ToIL-20 contained a low-complexity region (position 4–19) and an IL10 domain (position 35–172). ToIL-24 contained a signal peptide (position 1–28) and a SCOP domain d1lqsl_ (position 56–173). ToIL-27 contained a signal peptide (position 1–18) and two fibronectin type 3 (FN3) domains (positions 39–136 and 149–234). ToIL-34 contained an IL34 domain (position 1–161) and two low-complexity regions (position 170–192 and 236–250). Except for ToIL-24 and ToIL-27, all other ToIL proteins contained a typical IL domain.

3.2. Tertiary structures of ToILs

Protein function is defined by structure. Thus, homology modeling of

Table 2
Overall identity and pI values for the 12 ILs from *T. obscurus*.

Gene name	Number of amino acids	Molecular weight (kDa)	Isoelectric point (pI)	GenBank accession no.
ToIL-1β	256	29.05	4.98	OP351710
ToIL-1	349	39.55	5.05	OP351711
ToIL-6	227	25.61	7.02	OP351712
ToIL-10	227	25.53	8.23	OP351713
ToIL-11	201	23.27	9.12	OP351714
ToIL-12	210	23.33	6.16	OP351715
ToIL-17	158	17.65	10.20	OP351716
ToIL-18	189	22.10	5.08	OP351717
ToIL-20	176	20.24	8.07	OP351718
ToIL-24	182	20.75	5.96	OP351719
ToIL-27	252	27.78	8.96	OP351720
ToIL-34	261	29.80	8.96	OP351721

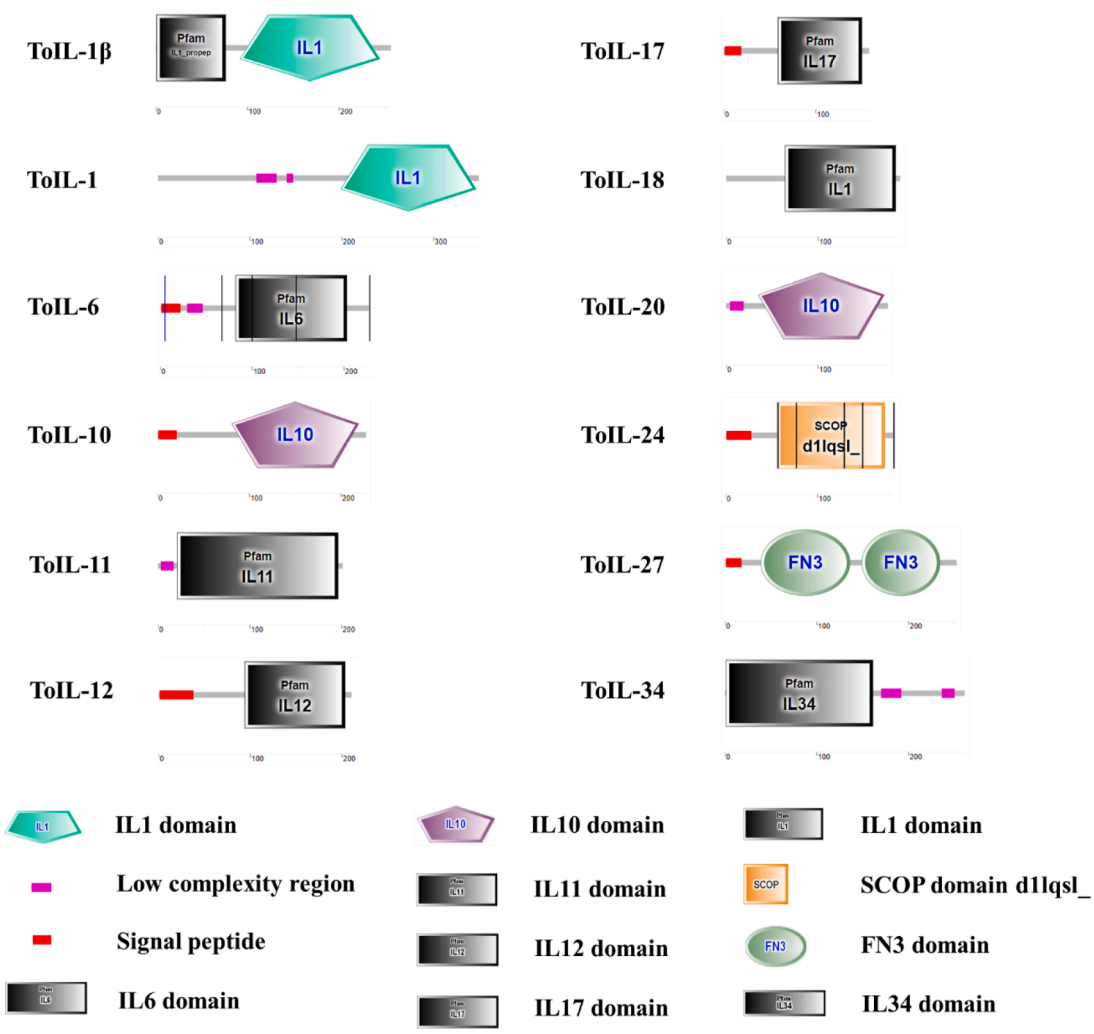

Fig. 1. Diagram of the domain architectures of 12 IL proteins from *T. obscurus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

12 ToILs tertiary structures was performed using the SWISS-MODEL algorithm (Fig. 2). The template structures used are shown in Table 3. ToIL-1β, ToIL-1, ToIL-6, ToIL-10, ToIL-11, ToIL-12, ToIL-17, ToIL-18, ToIL-20, ToIL-24, ToIL-27, and ToIL-34 were built up by using one, two, six, fourteen, six, five, one, one, six, seven, one, and nine α helices, and eight, five, zero, zero, zero, zero, seven, thirteen, two, zero, thirteen, and two β-sheets, respectively. The results indicated that these ToIL proteins were structurally similar to their corresponding IL homologs, implying the possible functional resemblance between the ILs of *T. obscurus* and other species.

3.3. Phylogenetic analysis

Based on the multiple alignment analysis, these 12 ILs from *T. obscurus* are lowly conserved. A phylogenetic tree was constructed to evaluate the molecular evolution relationship of 12 ToIL proteins of *T. obscurus* and other IL homologs from different species (Fig. 3). In the phylogenetic tree, 12 ToILs were clustered into different groups. ToIL-1β, together with three IL-1βs from *T. rubripes* (100.00% identity), *Takifugu bimaculatus* (95.70% identity), and *Takifugu flavidus* (95.31% identity), belong to one cluster. ToIL-1 is closely related to three IL-1 s from *T. rubripes* (99.71% identity), *T. bimaculatus* (97.43% identity), and *T. flavidus* (97.14% identity). ToIL-6 and two IL-6 s from *T. rubripes* (100.00% identity) and *T. flavidus* (97.16% identity) belong to one cluster. ToIL-10 and IL-10 from *T. nigroviridis* (61.08% identity) belong to one group. ToIL-11 and three IL-11 s from *T. rubripes* (100.00% identity), *T. flavidus* (98.91% identity), and *T. bimaculatus* (98.91% identity) belong to one clade. ToIL-12 and IL-12 from *T. rubripes* (100.00% identity) belong to one group. ToIL-17 and three IL-17 s from *T. rubripes* (100.00% identity), *T. flavidus* (95.57% identity), and *T. bimaculatus* (95.57% identity) belong to one clade. ToIL-18 and four IL-18 s from *T. rubripes* (100.00% identity), *T. bimaculatus* (90.00% identity), *T. nigroviridis* (65.22% identity), and *Labrus bergylta* (45.55% identity) were clustered into one group. ToIL-20 and IL-20 from *T. rubripes* (100.00% identity) belong to one cluster. ToIL-24 and two IL-24 s from *T. rubripes* (100.00% identity) and *T. bimaculatus* (99.21% identity) belong to one group. ToIL-27 and three IL-27 s from *T. rubripes* (100.00% identity), *T. bimaculatus* (99.21% identity), and *T. flavidus* (98.41% identity) belong to one cluster. ToIL-34 and two IL-34 s from *T. rubripes* (97.04% identity) and *T. flavidus* (83.25% identity) belong to one group.

3.4. Tissue distributions of ToIL genes

The mRNA expression of 12 *ToIL* genes was determined in six tissues of *T. obscurus* by using RT-qPCR. The results show that most *ToILs* were widely expressed in the heart, liver, kidney, spleen, gills, and intestine (Fig. 4). However, the expression levels of different *ToILs* differed. The transcripts of *ToIL-1β, ToIL-1, ToIL-11, ToIL-17, ToIL-18, ToIL-27*, and *ToIL-34* were detected mainly in the liver and heart. *ToIL-10* and *ToIL-12*

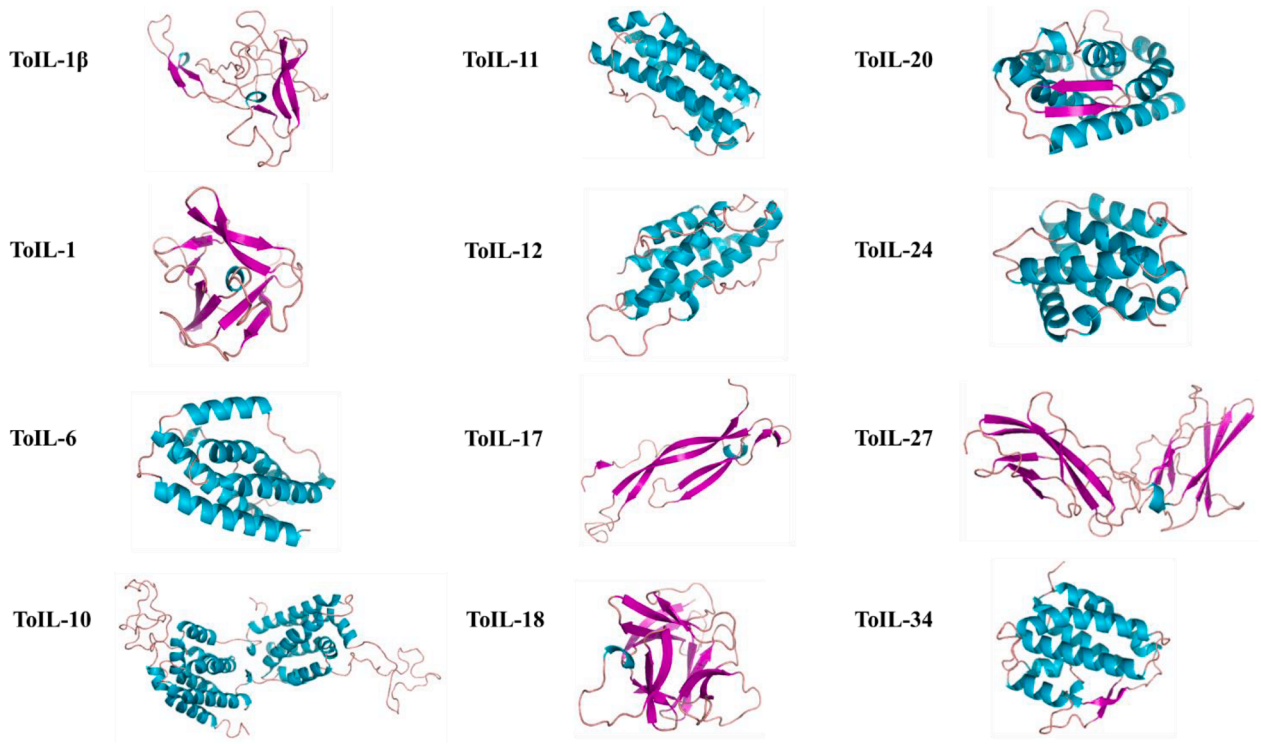

Fig. 2. Tertiary structures of 12 ToIL proteins from *T. obscurus*. Three-dimensional models of 12 ToILs were generated using SWISS-MODEL and PyMOL. The figure displays of the animated structure models, where the α-helices are colored blue, the β-sheets are colored purple, and the random coils are colored brown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Information on 3D model templates used in the study.

Protein name	Template name	SMTL ID	Sequence identity (%)	Oligo-State	GMQE	Citation
ToIL-1β	Interleukin-1 beta	3ltq.1.A	35.00	Monomer	0.31	[34]
ToIL-1	IL-1 beta	4 × 37.1.A	23.48	Monomer	0.17	unpublished
ToIL-6	Interleukin-6	7nxz.1.A	22.09	Monomer	0.45	[35]
ToIL-10	Interleukin-10	1ilk.1.A	29.33	Homo-dimer	0.46	[36]
ToIL-11	Interleukin-11	6o4o.1.A	28.75	Monomer	0.63	[37]
ToIL-12	Interleukin-12 subunit alpha	3hmx.1.B	28.48	Monomer	0.53	[38]
ToIL-17	Interleukin-17F	6hgo.2.A	33.04	Homo-dimer	0.45	[39]
ToIL-18	Interleukin-18	7al7.1.B	22.29	Monomer	0.54	[40]
ToIL-20	Interleukin-20	4doh.1.A	38.78	Monomer	0.69	[41]
ToIL-24	Interleukin 22	4o6k.3.A	31.11	Monomer	0.55	[42]
ToIL-27	Interleukin-27 subunit beta	7u7n.1.C	32.83	Monomer	0.54	[43]
ToIL-34	Interleukin-34	4exn.1.A	31.76	Homo-dimer	0.43	[44]

were primarily expressed in the heart and spleen. *ToIL-20* was mainly expressed in the liver and spleen. Other *ToILs* such as *ToIL-6* and *ToIL-24* showed the highest expression level in the spleen and intestine, respectively.

3.5. ToIL gene expression after bacterial infection

The roles of 12 *ToIL* genes in antibacterial innate immunity were investigated by observing the temporal mRNA expression of *ToILs* in the spleen and liver of *T. obscurus* upon challenge with *S. aureus* and *V. harveyi* by using RT-qPCR. The results showed that *S. aureus* and *V. harveyi* induced the expression of all *ToIL* genes in time-dependent manners in the tested tissues (Figs. 5 and 6). However, the maximum induction was observed at different times. In spleen, the *ToIL-1β* transcript reached the highest level at 12 h post *S. aureus* challenge (13.91-fold, $P < 0.01$) and at 24 h following *V. harbeyi* challenge (63.82-fold, $P < 0.01$, Fig. 5A). The expression of *ToIL-1* reached the peak at 72 h after stimulation by *S. aureus* (4.54-fold, $P < 0.01$) and at 12 h after *V. harbeyi* challenge (6.02-fold, $P < 0.01$, Fig. 5B). *ToIL-6* mRNA was quickly upregulated at 12 h post-injection of *S. aureus* (5.22-fold, $P < 0.05$) and *V. harveyi* (83.14-fold, $P < 0.001$, Fig. 5C). *ToIL-10* expression significantly increased at 72 h following *S. aureus* challenge (2.76-fold, $P < 0.01$) but lower after *V. harveyi* infection compared with uninfected controls, and the differences were not significant (Fig. 5D). The *ToIL-11* transcript reached the highest level at 72 h post *S. aureus* challenge (1.94-fold, $P < 0.01$) and at 12 h post *V. harbeyi* challenge (9.64-fold, $P < 0.01$, Fig. 5E). The *ToIL-12* expression was significantly upregulated at 72 h following *S. aureus* (8.49-fold, $P < 0.01$) and *V. harveyi* (5.96-fold, $P < 0.001$) challenges (Fig. 5F). *ToIL-17* expression was downregulated from 12 h to 72 h after *S. aureus* and *V. harveyi* infection (Fig. 5G). *ToIL-18* mRNA level was upregulated at 72 h after *S. aureus* infection (2.87-fold, $P < 0.001$) and then downregulated from 12 h to 72 h following *V. harbeyi* challenge (Fig. 5H). The *ToIL-20* transcript reached the highest level at 72 h post *S. aureus* challenge (3.86-fold, $P < 0.001$) and at 36 h post *V. harbeyi* challenge (5.77-fold, $P < 0.001$, Fig. 5I). The *ToIL-24* expression reached the peak at 24 h following *S. aureus* (5.15-fold, $P < 0.01$) and *V. harveyi* (3.73-fold, $P < 0.01$) challenge (Fig. 5J). The relative gene expression of *ToIL-27* significantly

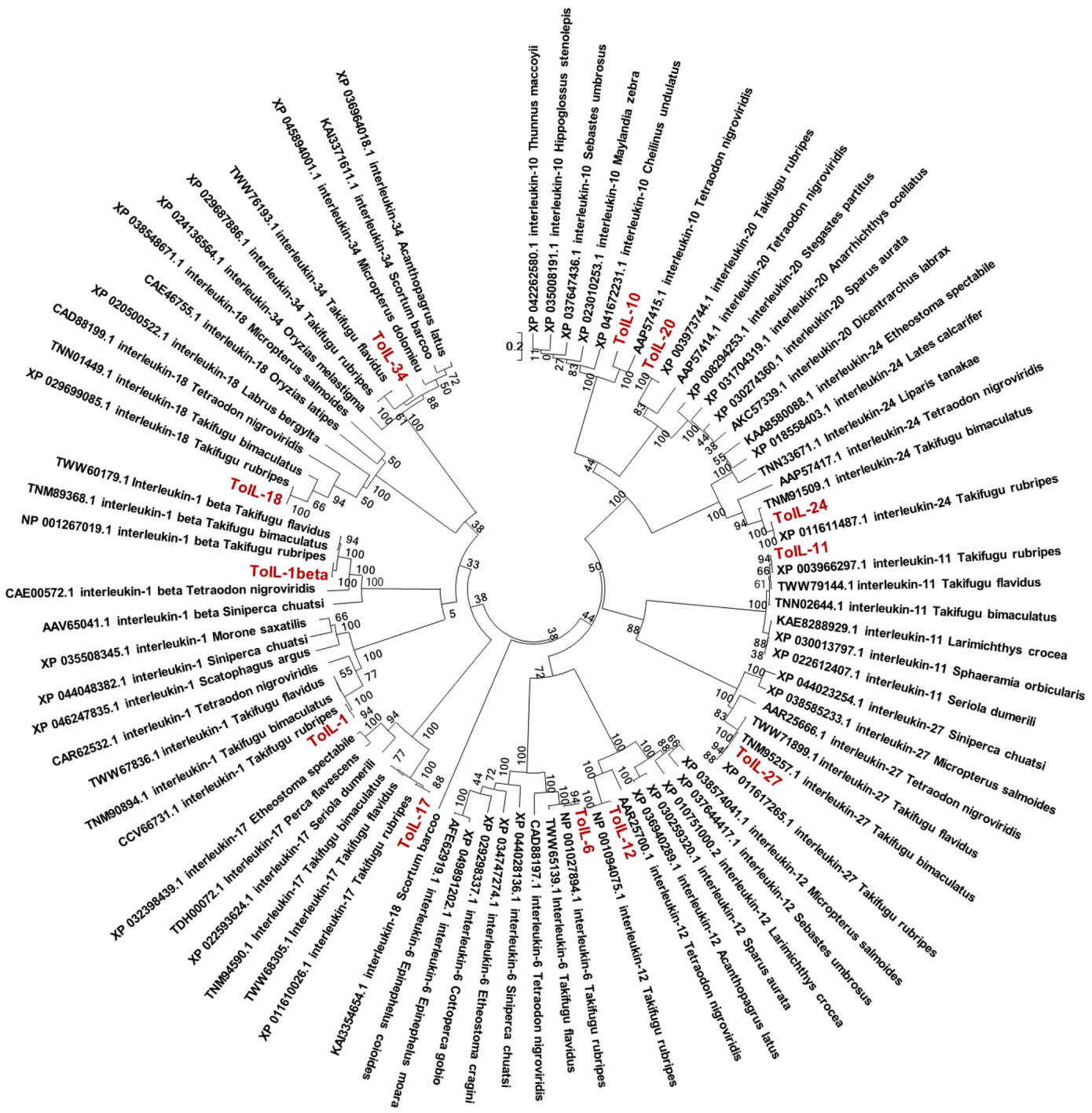

Fig. 3. Phylogenetic tree of ILs in *T. obscurus* and other species. The tree was constructed using the NJ method based on the amino acid alignments. Numbers were bootstrap values for 1000 trials. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased at 72 h upon *S. aureus* challenge (4.37-fold, $P < 0.001$) and at 36 h after *V. harveyi* challenge (2.81-fold, $P < 0.01$, Fig. 5K). The *ToIL-34* expression reached the peak at 72 h following *S. aureus* challenge (3.13-fold, $P < 0.01$) and at 48 h post *V. harveyi* challenge (10.74-fold, $P < 0.001$, Fig. 5L).

In liver, both *ToIL-1β* and *ToIL-6* expression reached the highest level at 12 h following *S. aureus* (14.09- and 14.91-fold, $P < 0.01$) and *V. harveyi* (20.19- and 57.03-fold, $P < 0.01$) challenge (Fig. 6A and C). The gene expression level of *ToIL-1* significantly increased at 72 h following *S. aureus* challenge (12.31-fold, $P < 0.001$) and at 24 h post *V. harveyi* challenge (15.01-fold, $P < 0.01$, Fig. 6B). The *ToIL-10* expression reached the peak at 12 h following *S. aureus* challenge (1.56-fold, $P < 0.01$) and at 24 h post *V. harveyi* challenge (2.68-fold, $P < 0.01$, Fig. 6D). The *ToIL-11* transcript reached the highest level at 12 h post *S. aureus* challenge (8.83-fold, $P < 0.01$) and at 36 h post *V. harbeyi* challenge (26.94-fold, $P < 0.01$, Fig. 6E). The transcript of *ToIL-12* was significantly upregulated at 36 h following *S. aureus* challenge (5.96-fold, $P < 0.01$) and at 24 h post *V. harveyi* challenge (5.41-fold, $P < 0.01$, Fig. 6F). *ToIL-17, ToIL-20*, and *ToIL-34* expression reached the highest level at 48 h upon *S. aureus* challenge (5.19-, 4.40-, and 4.91-fold, $P < 0.01$) and at 36 h after *V. harveyi* challenge (11.53-, 4.82-, and 5.56-fold, $P < 0.01$, Fig. 6G, I and L). The *ToIL-18* transcript reached

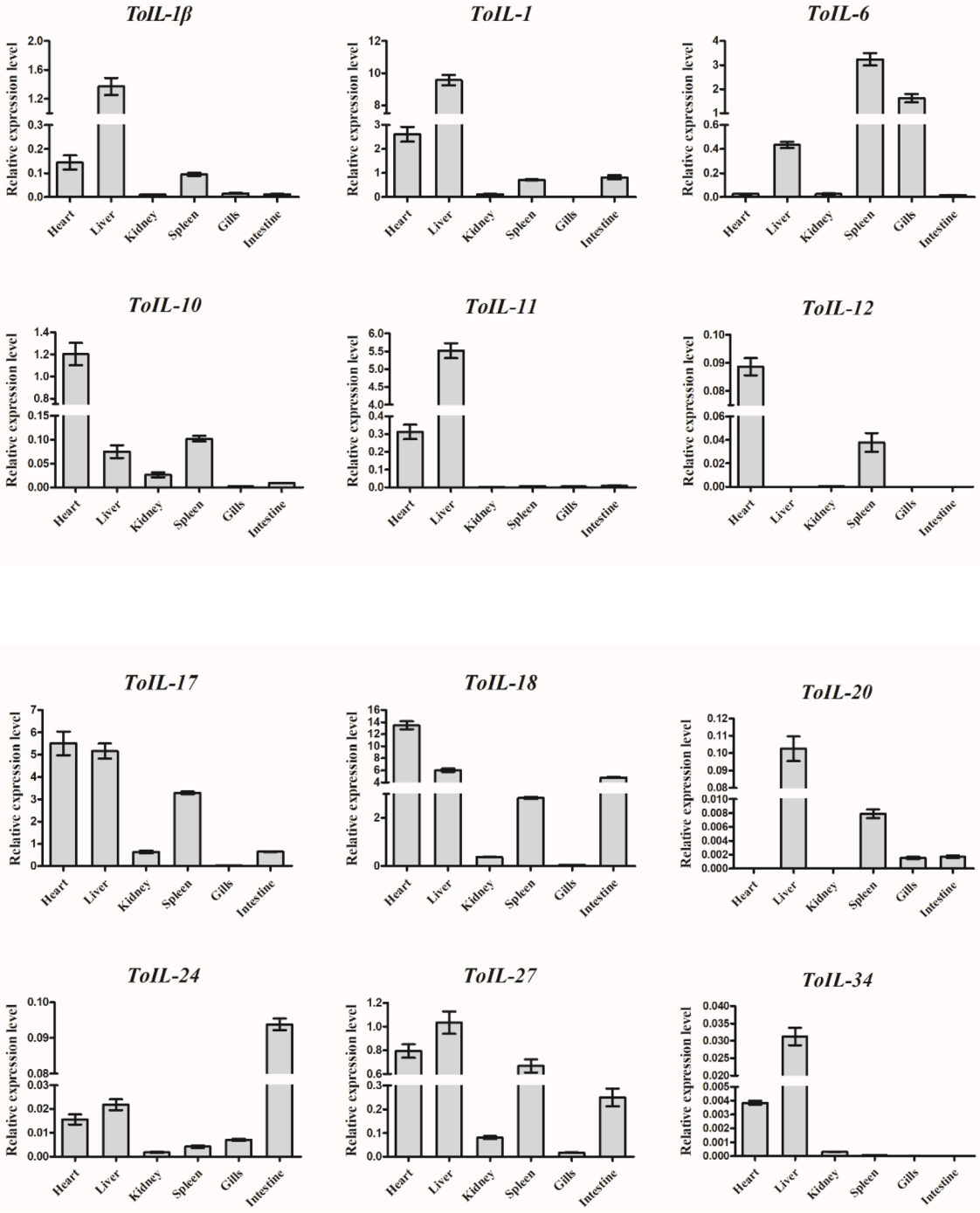

Fig. 4. Tissue distribution of 12 ILs in healthy *T. obscurus*. The transcripts of *ToIL-1β, ToIL-1, ToIL-6, ToIL-10, ToIL-11, ToIL-12, ToIL-17, ToIL-18, ToIL-20, ToIL-24, ToIL-27*, and *ToIL-34* were measured by RT-qPCR. *β-actin* was used as an internal control. The tissues, including heart, liver, kidney, spleen, gills, and intestine were collected from three individuals. Three biological repeats were set in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the pick at 72 h post *S. aureus* challenge (5.55-fold, $P < 0.001$) and at 36 h following *V. harbeyi* challenge (5.42-fold, $P < 0.001$, Fig. 6H). The maximum *ToIL-24* expression was reached at 12 h following *S. aureus* (4.98-fold, $P < 0.01$) and *V. harveyi* (4.01-fold, $P < 0.01$) challenge (Fig. 6J). The *ToIL-27* mRNA expression level decreased in the *V. harveyi* infection group but remarkably increased at 12 h after *S. aureus* infection (5.47-fold, $P < 0.01$, Fig. 6K). In addition, the transcripts of all *ToIL* genes in the spleen and liver did not change evidently after PBS challenge from 0 h to 72 h (data not shown).

4. Discussion

Fish innate immune systems have become an important barrier to the invasion of defense pathogens. ILs constitute an emerging family of cytokines and appear to be a distinct and potent signaling system involved in the control of immune response in fish [5]. In recent years, the functions of fish IL molecules have been extensively studied. The present study reports the identification and molecular characterization of the whole coding sequences of 12 ILs from *T. obscurus*, a teleost fish. The structural analysis of the 12 ToILs shared certain similar features

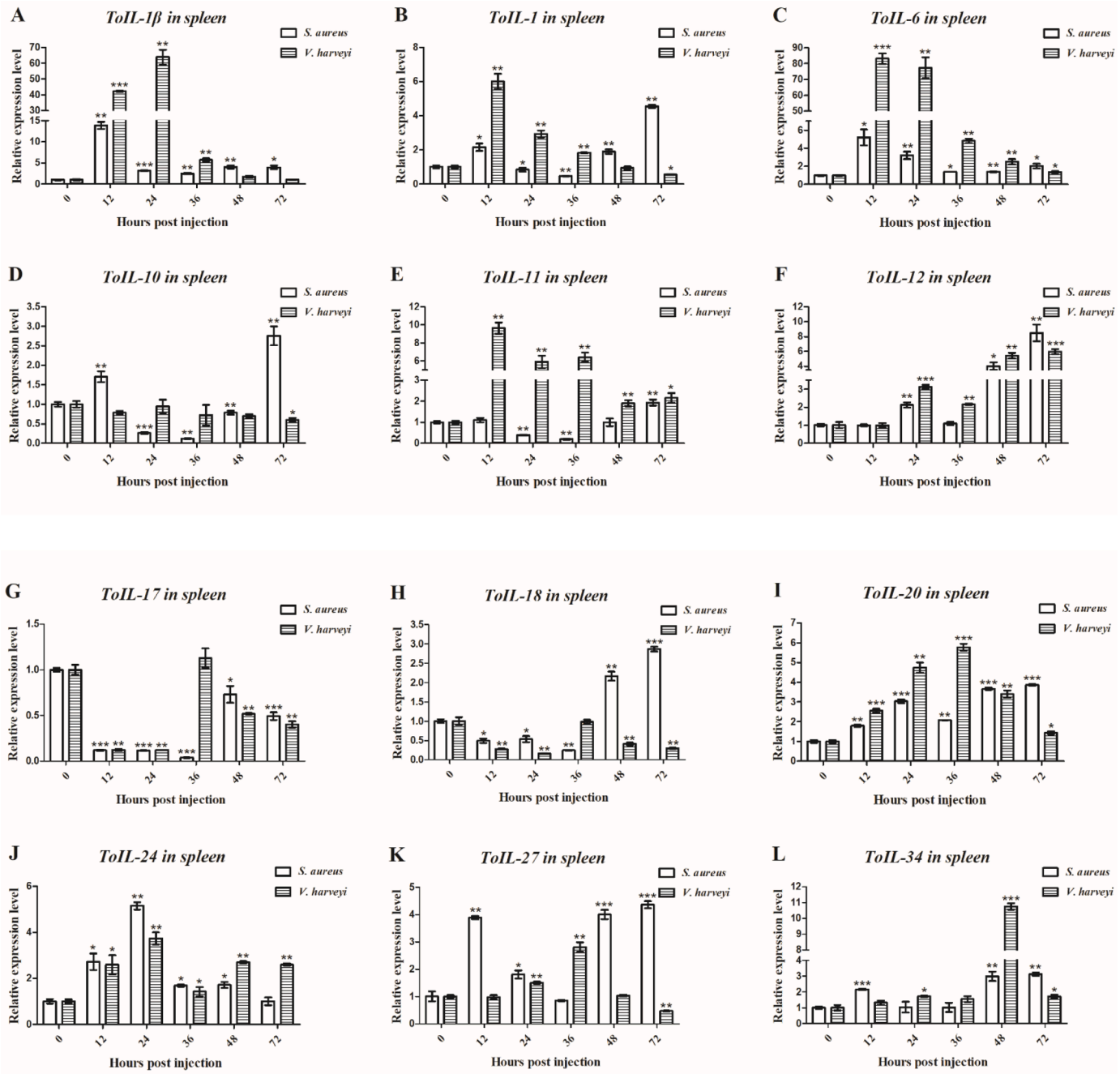

Fig. 5. Relative expression levels of 12 ILs in the spleen of *T. obscurus* at different time points (0, 12, 24, 36, 48, and 72 h) as measured with RT-qPCR after *S. aureus* and *V. harveyi* infection. *β-actin* was employed as an internal control. Each experiment was performed in triplicate. Data are shown as mean ± S.D. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with their mammalian orthologs. In addition to ToIL-24 and ToIL-27, the 10 other deduced ToIL proteins possessed a typical IL superfamily domain, which is consistent with the protein characteristics of ILs from other teleost fish. ToIL-27 contained two repeat units of FN3, which is a small autonomous folding unit present in many animal proteins involved in ligand binding. ToIL-6, ToIL-10, ToIL-12, ToIL-17, ToIL-24, and ToIL-27 proteins contained a signal peptide sequence, indicating that these molecules may be secreted through the classical pathway involving the golgi/endoplasmic reticulum route. ToIL-1, ToIL-6, ToIL-11, ToIL-20, and ToIL-34 also contained one or two low-complexity regions, which are protein sequences formed by a set of compositionally biased residues. Low-complexity regions are extremely abundant in cellular proteins and have also been reported in viruses, where they may partake in the evasion of the host immune system [45]. The conservative structures of ToILs implied their biological functions in innate immunity. In addition, the predicted ToIL proteins shared strong identities of 80%–100% with their counterparts in other selected vertebrates, and the phylogenetic tree and analysis of protein 3D structure both demonstrated that ToILs are evolutionarily conserved genes in animals and clear homologs of mammalian and fish ILs.

In general, the analysis of tissue distribution for genes provides a good understanding of their physiological roles. In *T. obscurus*, the expression of most *ToIL* molecules occurs constitutively in multiple tissues. The broad expression profiles of *ToILs* suggest that they are

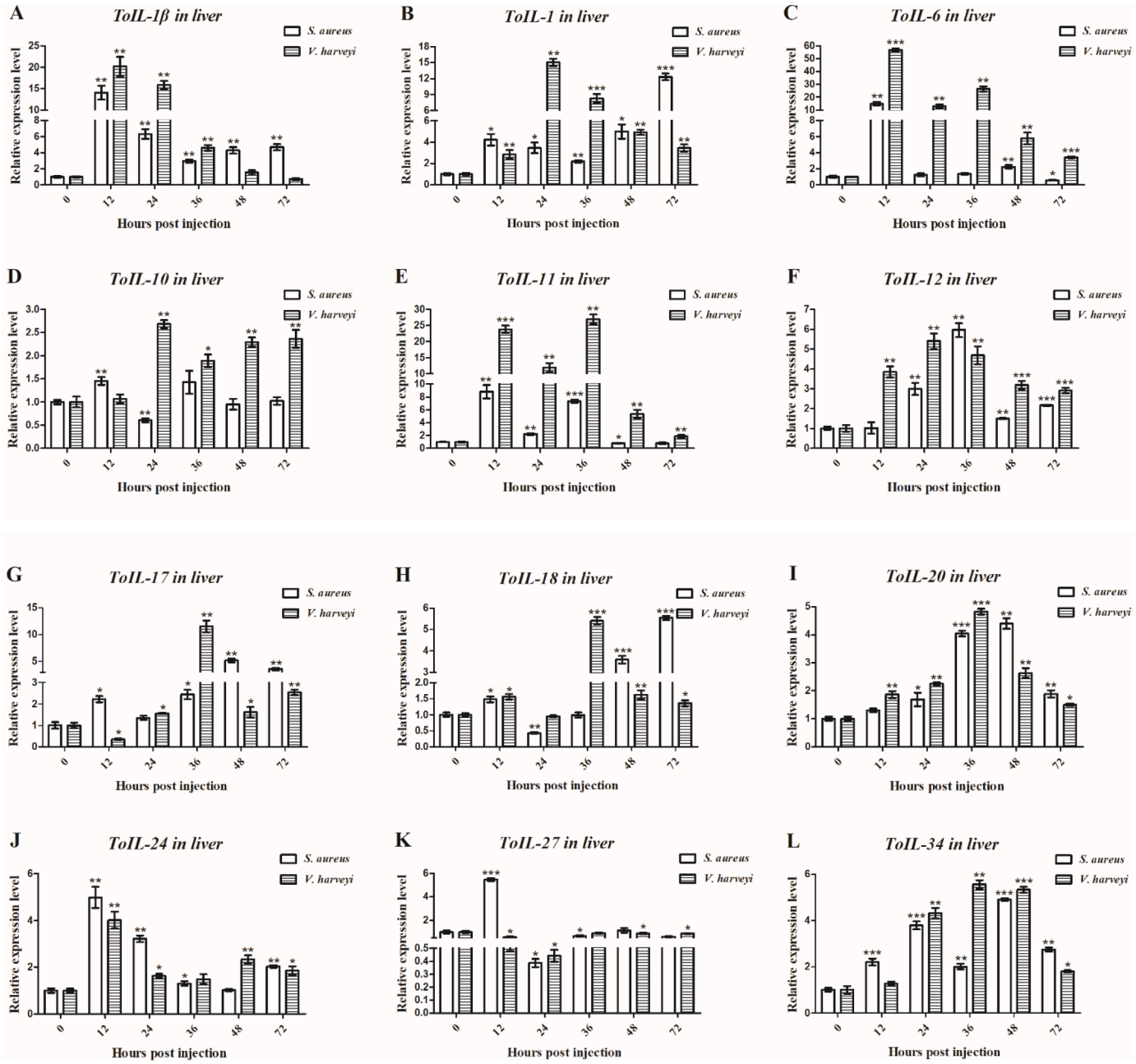

Fig. 6. Relative expression levels of 12 ILs in the liver of *T. obscurus* at different time points (0, 12, 24, 36, 48, and 72 h) as measured with RT-qPCR after *S. aureus* and *V. harveyi* infection. *β-actin* was employed as an internal control. Each experiment was performed in triplicate. Data are shown as mean ± S.D. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pleiotropic cytokines that may be required for tissue homeostasis. In fish, the expression profiles of *ILs* quite differ among species. The highest expression of *ToIL-1β, ToIL-1, ToIL-11, ToIL-20, ToIL-27*, and *ToIL-34* was detected in the liver, which consists of tissues with multifaceted functions in innate immunity, metabolism, and growth. In roughskin sculpin *Trachidermus fasciatus, rsIL-1β* gene was expressed most abundantly in the skin and moderately in the blood, heart, gills, and intestine [46]. *CsIL-11* from tongue sole *Cynoglossus semilaevis* was highly expressed in the blood, muscle, and heart [47]. In snakehead *Channa argus, shIL-20* was widely expressed, with the highest transcription in the liver and a relatively high transcription in the mucosal immune organs (gut and skin) [48]. In Japanese flounder *Paralichthys olivaceus, PoIL-34* expression is relatively high in the intestine and heart [49], and a relatively high *ToIL-6* expression was observed in the spleen. In *A. baeri*, a notably high level of *Abil-6* expression was reported in the spleen [20]. Spleen is regarded as the major primary lymphoid organ in fish, where monocytes/macrophages and lymphocytes reside, differentiate, and interact. The maximum expression of *ToIL-24* gene was detected in the intestine, which is a multifunctional organ central to both nutrient uptake, pathogen recognition, and regulation of the intestinal microbiome, suggesting a potential role in the mucosal immunity. A novel and unexpected finding in this study was that *ToIL-10, ToIL-12, ToIL-17*, and *ToIL-18* expression levels were very high in the heart, which possesses an extensive lymphatic network. *IL-10* from mandarin fish *Siniperca chuatsi* exhibited relatively high mRNA levels in the spleen, trunk kidney, head kidney, and intestine [50]. In rainbow trout, *IL-18A* and *IL-18B* were highly expressed in the gut, heart, and kidney [13]. The abundance of IL transcripts in different tissues differs across species possibly because of

the difference in the genetics and behavior and the culturing conditions of different fish samples.

As key members of cytokines, ILs play essential roles in infections and inflammatory diseases [6]. In the present study, the involvement of 12 *ToIL* genes in the immune response of *T. obscurus* was investigated by examining their expression in the liver and spleen of fish challenged with *S. aureus* (a Gram-positive bacterium) and *V. harveyi* (a Gram-negative bacterium). *S. aureus*, a metabolically flexible pathogen that causes infections in diverse settings, is increasingly being detected in various food-producing animals, including fish [51]. *V. harveyi*, which belongs to family *Vibrionaceae* of class *Gammaproteobacteria*, is one of the most serious pathogenic *Vibrio* in aquaculture [52]. The expression levels of all *ToIL* genes in the liver and spleen were significantly upregulated during infection with both pathogens, and their response varies over time. Similarly, the increase in *IL* expression was observed *in vivo* in other fish species treated with bacteria. In yellow catfish *Pelteobagrus fulvidraco*, the expression level of *Pf_IL-1β* was upregulated in the trunk kidney, head kidney, blood, spleen, heart, and liver after *Edwardsiella ictaluri* challenge [53]. Ayu *Plecoglossus altivelis PaIL-6* mRNA was significantly induced in the head kidney, liver, gills, and spleen upon *Vibrio anguillarum* infection [54], and gilthead seabream *sbIL-6* expression was remarkably upregulated in the head kidney by *V. anguillarum* challenge [18]. In golden pompano *T. ovatus, TOIL-10* and *TOIL-22* were rapidly activated after *Streptococcus agalactiae* SAΔphoB immunization and significantly increased to peak levels at 12 h and 4 days in the kidney and spleen, respectively, following challenge [24]. In crucian carp (*Carassius auratus gibelio*), the *IL-11* expression was enhanced in the kidney at early time points upon *Aeromonas hydrophila* infection [55]. After *V. anguillarum* stimulation, the mRNA expression of three *L. crocea ILs* (*lcIL12A, lcIL16* and *lcIL34*) was upregulated in the liver, spleen, and kidney [28]. In large yellow croaker, the expression of *LcIL-17C* and *LcIL-17D* significantly increased in the gills, head kidney, and spleen after *A. hydrophila* challenge [25]. In snakehead *C. argus*, injection with *Aeromonas schubertii* and *Nocardia seriolae* increased the *shIL-20* expression in the spleen and head kidney [48]. Grass carp *CiIL-34* expression was upregulated in the spleen and head kidney upon infection with *Flavobacterium columnare* [30]. The discrepancies in *IL* expression profiles in different species may have resulted from multiple factors such as the challenge routes (injection and bath), pathogen types, fish species, and age. Collectively, our results provided evidence that 12 *ToIL* genes are tightly associated with bacterial challenges and have great importance in host defense in *T. obscurus*.

In conclusion, the characterization of 12 *ToIL* genes from *T. obscurus* was described herein. ToILs possessed the characteristic structure features present in their respective homologs. Most *ToILs* were preferentially expressed in immune tissues such as liver, spleen, and intestine and increased significantly upon bacterial challenge. These data provide more detailed insights into the functional activities of the obscure puffer IL family. However, further in-depth studies are still needed to ascertain the functional role of fish ILs and their implication in immunity.

Ethics statement

The protocol was approved by the Ethics Committee of Experimental Animals at Hohai University.

Consent for publication

Manuscript is approved by all authors for publication.

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CRediT authorship contribution statement

Ying Huang: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Peng Luo:** Validation, Resources, Writing – review & editing. **Fu-Hui Jiang:** Methodology, Formal analysis, Software, Writing – original draft. **Hui-Ze Gao:** Formal analysis, Data curation, Software. **Li-Fan Cui:** Methodology, Software, Validation. **Zhe Zhao:** Conceptualization, Project administration, Resources, 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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2023.100103.

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