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Research paper

Molecular characterization and comparative expression analysis of two teleostean pro-inflammatory cytokines, *IL-1β* and *IL-8*, from *Sebastes schlegeli*

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ABSTRACT

Interleukin 1β (IL-1β) and interleukin 8 (IL-8) are two major pro-inflammatory cytokines which play a central role in initiation of inflammatory responses against bacterial- and viral-infections. IL-1β is a member of the interleukin 1 family proteins and IL-8 is classified as a CXC-chemokine. In the current study, putative *IL-1β* and *IL-8* counterparts were identified from a black rockfish transcriptomic database and designated as *RfIL-1β* and *RfIL-8*. The *RfIL-1β* cDNA sequence consists of 1140 nucleotides with a 759 bp open reading frame (ORF) which encodes a 252 amino acid (aa) protein, whereas the *RfIL-8* cDNA sequence (898 bp) harbors a 300 bp ORF encoding a 99 aa protein. Furthermore, the RfIL-1β aa sequence contains an IL-1 super family-like domain and an N-terminal IL-1 super family propeptide, while the amino acid sequence of RfIL-8 consists of a typical chemokine-CXC domain. Analysis of sequenced BAC clones containing *RfIL-1β* and *RfIL-8* showed each gene to contain 4 exons interrupted by 3 introns. Pairwise comparison and phylogeny analysis of these cytokine sequences clearly revealed their closer relationship with other corresponding members of teleosts compared to birds and mammals. Constitutive differences in *RfIL-1β* and *RfIL-8* mRNA expression were detected in a tissue-specific manner with the highest expression of each mRNA in spleen tissue. Two immune challenge experiments were conducted with *Streptococcus iniae* and polyinosinic:polycytidylic acid (poly I:C; a viral double stranded RNA mimic), and transcripts were quantified in spleen and peripheral blood cells. Significantly increased *RfIL-1β* and *RfIL8* transcript levels were detected with almost similar profile patterns, further suggesting a putative involvement of these pro-inflammatory cytokines in the rockfish immunity.

1. Introduction

Aquaculture in Korea has become a rapidly developing area because of its capability to match the growing demand for mariculture products among Asian countries. However, production losses due to pathogenic infections have become a major threat to the aquaculture industry (Lim, 2006). Thus, it is important to gain insights into fish host-defense mechanisms and putative factors in the fish immune system, that could be manipulated and used to protect fish from the major marine pathogenic infections mounted by bacteria, viral, fungi and parasites. Black rockfish (*Sebastes schlegeli*) is the second most largely cultured fish species in South Korea. Its aquaculture has been mainly affected by a combination of bacterial species (Han et al., 2011; Kang et al., 2004).

IL-1β and IL-8 are generally described as pro-inflammatory cytokines due to their functional impingement in initiation of inflammatory mechanisms (Dinarello, 1997; Petering et al., 1999). More specifically, IL-8 is further categorized as a chemokine (J. Kerry, 2004) due to its

Abbreviations: IL-1β, interleukin 1 beta; IL-8, interleukin 8; Rf, Rockfish; cDNA,, complementary Deoxyribonucleic Acid; bp, base pair; ORF, open reading frame; Aa, amino acid; IL-1α, interleukin 1 alpha; ICE, interleukin-1-converting enzyme; ELR, glutamic acid-leucine–arginine; RNA, ribonucleic acid; RIN, RNA integration score; DNA, deoxyribonucleic acid; BAC, bacterial artificial chromosome; NCBI, National Center for Biotechnology Information; MSA, multiple sequence alignments; SMART, Simple Modular Architecture Research Tool; NJ, neighbor joining; MEGA, molecular evolutionary genetic analysis; CDD, conserved domain database; PDB, protein data base; *S. iniae*, *Streptococcus iniae*; PBS, phosphate buffered saline; poly I:C, polyinosinic:polycytidylic acid; qPCR, quantitative real-time PCR; PCR, polymerase chain reaction; RfEF1A, Black Rockfish Elongation Factor-1 Alpha; SD, standard deviation; UTR, untranslated region; kDa, *Kilodalton*; D-X-D, aspartic acid residue and X, any hydrophobic residue; ICE, cutting site for caspase 1; 3D, three dimensional; mRNA, messenger ribonucleic acid; LPS, lipo-polysaccharide; EPS, extracellular polysaccharides; CFU, colony forming unit; μl, microliter; U, units; dNTPs, deoxynucleotide Triphosphates; °C, degrees of Celsius; s, seconds; Ng, nanogram; Rpm, revolutions per minute; h, hours; Nm, nanometers.

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specific involvement in chemotactic motions in inflammatory reactions. According to the previous studies on mammals, IL-1β is categorized under IL-1 family proteins (Dinarello, 1997). At present, all the identified and annotated counterparts within this family are divided into three major groups; IL-1α, IL-1β and IL-1 receptor antagonists (Smith et al., 2000). IL-1β, mainly produced by activated macrophages and monocytes (Dinarello, 1997), is also considered as a pleiotropic cytokine due to its diverged functions in various molecular and immunological processes (Bird et al., 2002). Moreover, role of IL-1β ranges from pro-inflammatory to anti-inflammatory behavior (Apte et al., 2006; Dinarello, 1996). IL-1β plays a major role as an intracellular mediator of immune responses against specific pathological conditions and acts as a regulator of the intensity of corresponding immune responses (Apte et al., 2006). On the other hand, IL-1β has the ability to stimulate the proliferation and differentiation of immune-competent cells (Bird et al., 2002). Structurally, IL-1β family cytokines have a "β-trefoil structure" which is composed of 12 β-sheets (Hughes, 1994). IL-1β is produced as an inactive precursor protein and subsequent cleavage at the conserved cutting site by the caspase-1 enzyme (interleukin-1-converting enzyme, ICE) activates the IL-1β cytokine. The resulting mature IL-1β peptide subsequently participates in inflammatory reactions (Dinarello, 1997).

Mammalian IL-8 counterparts identified to date are small cytokine molecules (~99aa) belong to CXC chemokine family proteins (Baggiolini et al., 1989; Zlotnik and Yoshie, 2000), and are collectively designated as CXCL8 (Bird et al., 2002). CXC chemokines are generally characterized by the presence of a CXC signature motif. CXC cytokines can be further divided into two subgroups, ELR + CXC subgroup and ELR − CXC subgroup, depending on the presence or absence of a glutamic acid-leucine–arginine (ELR) tripeptide sequence. These structural characteristics are known to influence functionality. Based on the presence of ELR motif, IL-8 is categorized under the ELR + CXC subgroup (J. Kerry, 2004). IL-8 is produced by a range of cell types such as macrophages, lymphocytes, peripheral blood mononuclear cells and epithelial cells (Goodman et al., 1991; Gregory et al., 1988; Mukaida et al., 1998; Nakamura et al., 1991). Functionally, IL-8 cytokine is well known as a recruiting agent of neutrophils and other important leukocytes at the site of inflammatory reactions (Li and Yao, 2013). IL-8 is also involved in promoting angiogenesis and causing oxidative burst in neutrophils (Zeilhofer and Schorr, 2000).

Most of the IL-1β and IL-8 counterparts from many teleosts have been examined in order to assess their functional involvement in innate immunity as well as to monitor the expression pattern upon pathogenic infections. To date, characterization and expression analysis of *IL-1β* counterpart has been reported from teleost species including *Oreochromis niloticus* (Lee et al., 2006), *Dicentrarchus labrax* (Buonocore et al., 2005; Scapigliati et al., 2001), *Sparus aurata* (Pelegrin et al., 2001), *Trachidermus fasciatus* (Liu et al., 2012), and *Epinephelus coioides* (Lu et al., 2008). Currently, there is a trend of investigating the possibility of manipulating pro-informatory cytokines, such as IL-1β, as an immune adjuvant in vaccination (Buonocore et al., 2004; Min et al., 2001). Similarly, different types of studies have been undertaken on IL-8 counterpart in order to demonstrate the information at molecular level on immunological basis. To date, *IL-8* counterparts from teleost species such as *Ctenopharyngodon idellus* (Wang et al., 2013), *Aristichthys nobilis* (Li et al., 2013), *Scophthalmus maximus* (Hu et al., 2011), and *Larimichthys crocea* (Li and Yao, 2013) have been characterized. Pro-inflammatory functions of *IL-1β* and *IL-8* genes, along with their expression patterns, have been studied (Corripio-Miyar et al., 2007; Seppola et al., 2008; Sigh et al., 2004). Even though, several studies have performed with individual interleukins, not many studies are available demonstrating the expressional relationship of pro-inflammatory cytokines under pathological conditions.

In the current study, we identified the cDNA sequences of black rockfish IL-1β (*RfIL-1β*) and IL-8 (*RfIL-8*) from a transcriptomic database, obtained corresponding gene sequences by screening a genomic library, and characterized resulting sequence data using bioinformatics tools. Moreover, mRNA expression patterns of the two genes were investigated in different tissue types of healthy black rockfish. Further, *RfIL-1β* and *RfIL-8* mRNA expressions in spleen tissue and blood cells were documented after induction of specific pathological conditions in a comparative manner.

2. Material and methods

2.1. Construction of black rockfish cDNA library and sequence identification

The 454 GS FLX sequencing technique (Droege and Hill, 2008) was applied to the construction of black rockfish cDNA sequences database. Total RNA extraction was carried out with blood, liver, gill and spleen tissues of black rockfish. The extracted RNA was then cleaned using RNeasy Mini kit (Qiagen, USA) and assessed for the quality and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), keeping an RNA integration score (RIN) of 7.1. Subsequently, RNA was fragmented into fragments with a mean size of 1147 bp using the titanium system (Roche 454 Life Science, USA). Finally, sequencing was carried out on half a picotiter plate on a Roche 454 GS FLX DNA platform at Macrogen, Korea. The raw 454 reads were trimmed to remove adaptor and low-quality sequences, and *de novo* assembled into contigs using GS Assembler (Roche 454 Life Science, USA) with the default parameters. The cDNA sequences of *RfIL-1β* and *RfIL-8* were identified from the established black rockfish cDNA sequence database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Black rockfish genomic library construction and PCR screening

Using black rockfish genomic DNA, a randomly sheared bacterial artificial chromosome (BAC) library was custom constructed (Lucigen, USA). A series of PCRs were carried out with sequence specific primer pairs (Table 1) in order to identify clones containing full-length *RfIL-1β* and *RfIL-8* genomic sequences according to the manufacturer's instructions. Positive clones were located as described elsewhere (Umasuthan et al., 2014). Two individually identified BAC clones, one containing a full-length *RfIL-1β* and one containing a full-length *RfIL-8* gene, were sequenced by GS-FLX™ system (Macrogen, Korea). Nucleotide sequences of the *RfIL-1β* (Accession No: KP069026) and *RfIL-8* (Accession No: KP069025) genes were deposited in NCBI GenBank.

2.3. Sequence characterization of RfIL-1β and RfIL-8

The open reading frame (ORF) and amino acid (aa) sequences of RfIL-1β and RfIL-8 were predicted using DNAssist 2.2. The orthologous protein sequences of RfIL-1β and RfIL-8 obtained by the BLAST search program were separately compared. Subsequent analysis of protein sequences was carried out with the EMBOSS needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) for pairwise comparison and the program ClustalW2 (Thompson et al., 1994) for multiple sequence alignments (MSA), respectively. The evolutionary relationship of RfIL-1β and RfIL-8 with their orthologs were obtained by generating phylogenetic tree using the MEGA software version 5 (Tamura et al., 2007), based on the Neighbor-Joining (NJ) method with the support of

Table 1
Oligomers used in this study.

Gene	Primer sequence (5′-3′)	BAC screening/qPCR amplification
RfIL-1β	GTGGCACCACAAAAGTTCACCTGAA	BAC screening forward forward
	CTTGCGGCAACACAGGTAGAGATTTG	BAC screening forward reverse
RfIL-8	ATTGTGGTGCTCCTGGCTTTCC	BAC screening forward forward
	GGCAAACCTCTTGGCCTGTCTTT	BAC screening forward reverse
RfEF-1A	AACCTGACCACTGAGGTGAAGTCTG	qPCR amplification forward
	TCCTTGACGGACACGTTCTTGATGTT	qPCR amplification reverse

bootstrapping values taken from 5000 replicates. Information on the characteristic signature domains in protein sequences were predicted by the ExPASy Prosite server (http://prosite.expasy.org) and the NCBI conserved domain database (CDD) (Marchler-Bauer et al., 2011).The tertiary structures of RfIL-1β and RfIL-8 were predicted using online SWISS model interface (Arnold et al., 2006). A 1.58 Å resolution crystal structure of chicken cytokine IL-1β (PDB no. 2WRY) and a 2.00 Å in solution structure of human IL-8 (PDB no. 3il8.1A) were selected from the PDB database and used as the templates for IL-1β and IL-8, respectively. Three-dimensional (3D) images were visualized and analyzed using PyMOL molecular graphic software version 1.3 (DeLano, 2002).The genomic sequences of *RfIL-1β* and *RfIL-8* were annotated using NCBI-Spidey server (http://www.ncbi.nlm.nih.gov/spidey/) and the respective diagrams were generated using GeneMapper (2.5) software to illustrate the exon-intron arrangements. Previously discovered *IL-1β* and *IL-8* genomic sequences were obtained from NCBI GenBank database and compared with *RfIL-1β* and *RfIL-8* genomic DNA sequences.

2.4. Experimental animals and tissue collection

Healthy black rockfish with an average size of 200 g were pre-acclimatized to the laboratory conditions in the aquariums of The Vaccine Research Centre of Jeju National University, Jeju Self-Governing Province, Republic of Korea. At the first step of tissue collection, blood (~1 ml) was collected from five fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA) and the peripheral blood cells were separated by an immediate centrifugation at 3000 ×*g* for 10 min at 4 °C. Then, other tissues including head kidney (a distinct hematopoietic/lymphatic organ found in some fish), spleen, liver, gill, intestine, kidney, brain, muscle, skin, heart, stomach, testis and ovary were dissected from five healthy fish. All the excised tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C.

2.5. Immune challenge experiments

The immune responsiveness of two rockfish genes, *RfIL-1β* and *RfIL-8*, was monitored by performing two immune challenge experiments. *Streptococcus iniae* (obtained from Department of Aqualife Medicine, Chonnam National University, Korea) and poly I:C (Sigma, USA) were used as pathogenic stimuli in the experiment. Thirty-five fish were intraperitoneally injected with 200 μl of poly I:C (1.5 μg/μl in PBS) and another 35 fish were likewise injected with *S. iniae* (200 μl 10^5 CFU/μl in PBS). As an injection control, another group of 35 fish was intraperitonially injected with 200 μl of PBS. Thereafter, five animals from each injected group and five animals from an un-injected control group were sacrificed for spleen and blood collection at each of the following post-injection periods: 3, 6, 12, 24, 48, and 72 h as described in Section 2.4.

2.6. RNA extraction and cDNA synthesis

At the end of each post-injection incubation period, five fish from each of the experimental groups (poly I:C-injected, *S. iniae*-injected, PBS-injected, and un-injected), were sacrificed. Blood from the five sacrificed poly I:C injected fish was pooled as was spleen tissue (~40 μg tissue from each fish per pool). Separate pooling of blood and spleen tissues was repeated for the other four experimental groups (*S. iniae*-injected, PBS-injected, and un-injected). After blood and spleen pools were prepared for each experimental group at each post-injection incubation period, total RNA was extracted from each pool using QIAzol® (Qiagen) following the vendor's protocol. First-strand cDNAs were synthesized using the PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Japan) following the manufacturer's instructions.

2.7. Transcriptional profiling under physiological conditions and pathogenic stress

Quantification of the mRNA level was carried out using all the extracted tissues mentioned above in Section 2.4 and Section 2.5 by quantitative PCR (qPCR) techniques. The qPCR was carried out using the Dice™ TP800 Real-Time Thermal Cycler System (TaKaRa) in 15 μl reaction volume containing 4 μl of diluted cDNA, 7.5 μl of 2× TaKaRa Ex*Taq*™ SYBR premix, 0.6 μl of sequence specific primers (Table 1), and 2.3 μl of reaction H_2O. The following thermal cycling conditions were used: 10 s at 95 °C, followed by 35 cycles of 5 s at 95 °C, 10 s at 58 °C and 20 s at 72 °C, and a final cycle of 15 s at 95 °C, 30 s at 60 °C and 15 s at 95 °C. The Dice™ Real-Time System Software (version 2.0) automatically set the baseline. The mRNA expression of *RfIL-1β* and *RfIL-8* was determined by the Livak method (Livak and Schmittgen, 2001). The same qPCR profile was used for detection of the internal control gene, black rockfish elongation factor-1 alpha (RfEF1A; KF430623) expression using gene specific primers (Table 1). Expression levels were analyzed in triplicates and the data were presented as mean ± standard deviation (SD) of relative mRNA expression. The relative expression level of *RfIL-1β* and *RfIL-8* at the 0 h time point (un-injected control) was used as the baseline for comparison. Moreover, expression levels were further normalized to the corresponding PBS-injected controls at each time point.

2.8. Statistical analysis

For comparison of relative *RfIL-1β* and *RfIL-8* mRNA expression, the two-tailed un-paired t-test was used. The significant differences of experimental groups and un-injected control group were defined as $P < 0.05$.

3. Results

3.1. Computer based sequence characterization and phylogenetic relationship

The *RfIL-1β* and *RfIL-8* cDNA sequences were isolated from a Black rockfish transcriptome database. The *RfIL-1β* cDNA sequence was 1140 nucleotides in length and contained a 759 bp ORF that encodes for a sequence of 252 aa. Lengths of the 5′ and 3′ un-translated regions (UTRs) were 25 bp and 356 bp, respectively. The predicted molecular mass of the putative protein was 28 kDa and the theoretical isoelectric point was 5.0. The complete cDNA sequence (898 bp) of *RfIL-8* was comprised of a 300 bp ORF encoding a peptide of 99 aa, with a predicted molecular mass of 11 kDa and a theoretical isoelectric point of 9.2. According to NCBI CDD and ExPASy Prosite prediction, the putative RfIL-1β aa sequence contains an IL-1 propeptide domain (14–77 aa) and an IL-1 superfamily signature domain (95–235 aa). Meanwhile, the aa sequence of RfIL-8 was found to bear a signal peptide (1–23 aa), followed by a typical chemokine CXC domain (32–95 aa), while no signal peptide was detected in the RfIL-1β aa sequence.

In MSA (Table 2 and Fig. 1), RfIL-1β was observed to harbor two conserved domains namely a IL-1 propeptide domain and a IL-1 superfamily signature domain (Fig. 1A) as found in roughskin sculpin, yellow perch and Antarctic icefish. Moreover, high level of sequence identity was observed within the areas where β-sheets are gathered to form the β-tetroid structural feature of IL-1 family proteins. The MSA of IL-8 orthologs identified a functionally important chemokine CXC domain and its conservation among the examined vertebrate counterparts (Fig. 1B). Pairwise sequence alignment exhibited that roughskin sculpin (*Trachidermus fasciatus*) IL-1β shares the highest identity (69.1%) and similarity (77.0%) with RfIL-1β whereas RfIL-8 showed 85.9% identity and 90.9% similarity with the European seabass (*Dicentrarchus labrax*) counterpart (Table 2). From the phylogenetic analysis carried out with different IL-1β aa sequences, including RfIL-1β, three main clusters were obtained (mammalian, avian and teleosts), where RfIL-1β

Table. 2
Amino acid identity and similarity of RfIL-1β and RfIL-8 entire sequences to other homologs.

Gene	Species name	Common name	Accession no	Amino acid	Identity%	Similarity%
RfIL-1β	*Trachidermus fasciatus*	Roughskin sculpin	AFH88676	256	69.1	77.0
	Perca flavescens	Yellow perch	AGL42376	254	67.3	77.6
	Epinephelus coioides	Orange-spotted grouper	ABV02593	261	62.8	73.2
	Chionodraco hamatus	Antarctic icefish	CAD92853	247	62.2	76.1
	Lateolabrax japonicas	Japanese seabass	AAQ89601	265	60.8	72.5
	Oplegnathus fasciatus	Barred knifejaw	ACH87392	267	57.7	69.3
	Tetraodon nigroviridis	Spotted green pufferfish	CAE00572	264	56.8	67.8
	Takifugu rubripes	Fugurubripes	NP_001267019	262	52.7	69.5
	Gallus gallus	Chicken	NP_989855	267	25.6	39.5
	Mus musculus	Mouse	NP_032387	269	21.1	36.1
	Homo sapiens	Human	P01584	269	21.0	34.8
RfIL-8	*Dicentrarchus labrax*	European seabass	CAM32186	99	85.9	90.9
	Latris lineata	Striped trumpeter	ACQ99511	99	85.9	91.9
	Anoplopoma fimbria	Sablefish	ACQ57874	99	80.8	90.9
	Lutjanus sanguineus	Humphead snapper	AGV99968	96	77.8	83.8
	Takifugu rubripes	Fugurubripes	NP_001027759	98	75.8	85.9
	Paralichthys olivaceus	Japanese flounder	BAN04720	98	65.7	83.8
	Danio rerio	Zebrafish	XP_001342606	98	55.6	71.7
	Oncorhynchus mykiss	Rainbow trout	AAO25640	97	54.5	75.8
	Gallus gallus	Chicken	NP_990349	104	40.6	61.5
	Alligator sinensis	Chinese alligator	XP_006018818	104	38.7	61.5
	Xenopuslaevis	African clawed frog	AEB96252	103	37.1	63.1
	Homo sapiens	Human	NP_000575	99	33.7	57.6
	Bos Taurus	Cattle	NP_776350	101	31.1	54.5

clustered together with other fish species (Fig. 2). As expected, phylogenetic analysis located the RfIL-8 within teleost clade (Fig. 2). Corresponding counterparts from mammals and birds were separately clustered together. However, previously identified IL-8-like counterparts such as teleost-specific IL-8-like molecule (*Cyprinus carpio* CXCa) and direct ortholog of mammalian IL-8 (*C. carpio* IL-8) from *C. carpio* were grouped with teleost counterparts and mammalian counterparts, respectively (Abdelkhalek et al., 2009).

3.2. Molecular modeling of tertiary structures of RfIL-1β and RfIL-8

The crystal structure of chicken IL-1β, which shared 31.79% sequence identity with putative RfIL-1β sequence, was selected as the template. The structural QMEAN4 value for model quality was −6.97. Derived structure for RfIL-1β (Fig. 3A) exhibited β-trefoil conformation composed of twelve β-sheets (aa positions; 93–100, 103–110, 113–118, 131–133, 147–155, 159–166, 169–176,201–204,211–215, 222–224, 234–236 and 246–249) and two α-helixes (detected within 7th and 8th β-strands), which is a signature conformation for IL-1 family proteins. The solution structure of the IL-8 dimer was used as the template to obtain the monomeric 3D confirmation of RfIL-8 (Fig. 3B), in which, selected template shared 40.3% of sequence identity with RfIL-8. The structural QMEAN4 value for model quality was −1.32 In modeled RfIL-8 structure, three anti-parallel β-strands (aa position 50–54, 65–70 and 75–79) and a C-terminal α-helix (aa position 87–94) were identified. The RfIL-8 dimer is depicted based on its monomeric structure (Fig. 3C).

3.3. Genomic architectural analysis of RfIL-1β and RfIL-8

Genomic sequences of *RfIL-1β* and *RfIL-8* have revealed the information regarding the intron–exon arrangements and coding sequences. In *RfIL-1β* coding sequence, four exons were split out by three introns (Fig. 4A). Similarly, in *RfIL-8* genomic DNA sequence, four exons were interrupted by three introns (Fig. 4B). In our comparative analysis of different *IL-1β* genomic DNA sequences, only a few compared fish orthologs possessed similar number of intron-exon combinations, while most of the others harbored different numbers of intron-exon combinations. The last three exons in the coding sequences are almost conserved among fish counterparts as well as in other mammalian genomic sequences compared. However, initial portion of fish IL-1β proteins is encoded by variable number of exons in a species-specific manner. In contrast, number of exons and introns in coding regions of mammalian *IL-1β* seems to be conserved in examined species.

3.4. Tissue-specific mRNA expression profiles of RfIL-1β and RfIL-8

In order to investigate the distribution of *RfIL-1β* and *RfIL-8* mRNA expression in different tissues under the physiological conditions, qPCR was carried out with gene specific primers. Both *RfIL-1β* and *RfIL-8* exhibit a diverse range of mRNA expression among all the investigated tissues with a differential prominence in different tissues (Fig. 5). In the *RfIL-1β* expression profile, the highest expression level was detected in spleen tissue followed by kidney and skin tissues. Moderate levels of mRNA expressions were detected in head kidney, gill, liver, testis, and intestine. Lower levels of expressions were detected in rest of the tissues. Intriguingly, the expression patterns of *RfIL-8* reflected those observed for *RfIL-1β*, spleen expressed the highest amount of *RfIL-8* transcripts, followed by gill and skin. Moderate expression levels of *RfIL-8* were observed in intestine, blood, brain, head kidney and kidney and lower levels were observed in remaining tissues.

3.5. Comparative investigation on transcriptional responses upon immune stimulation

Both *RfIL-1β* and *RfIL-8* genes exhibit prominent induction in response to *S. iniae* and poly I:C challenges. As shown in Fig. 6A, spleen tissues of *S. iniae*-injected fish showed their highest *RfIL-1β* and *RfIL-8* mRNA induction at 12 h p.i., whereas the most pronounced inductive response upon poly I:C challenge was detected at 6 h p.i. Moreover, induction of both *RfIL-1β* and *RfIL-8* in the spleen of *S. iniae*- and poly I:C-challenged animals show a similar peak and decay pattern over time (Fig. 6A). In blood cells of challenged animals (Fig. 6B), *S. iniae* gradually and significantly induced the *RfIL-1β* mRNA level, while triggering high level *RfIL-8* expression from 6 h–72 h, except at 24 h p.i. Meanwhile, poly I:C elicited an increased level of both *RfIL-1β* and *RfIL-8* at the mid-phase, and a down-regulation was noted at the later phase, as observed in spleen.

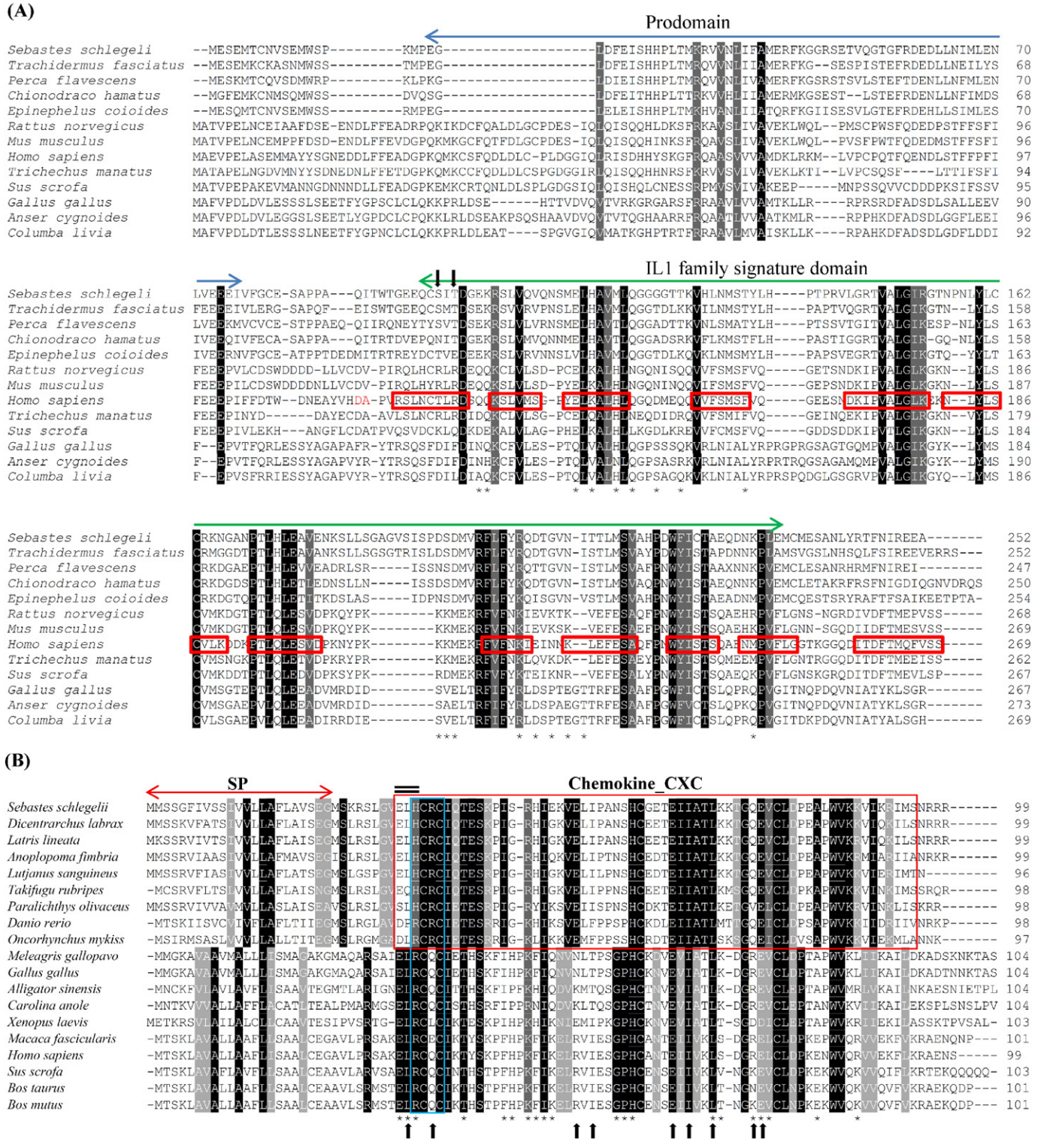

Fig. 1. Clustal W multiple sequence alignments of the deduced amino acid sequences of RfIL-1β (A) and RfIL-8 (B) with known IL-1β and IL-8 proteins. The identical residues are highlighted with black color and semi conservative residues are highlighted with gray color. Receptor binding sites are indicated with asterisks. (A) Prodomain and IL-1 family signature are indicated within blue- and green color double headed arrows, respectively. Mammalian ICE cutting site is indicated on human IL-1β in red color letters. Highly conserved β-strand forming residues are marked with red color boxes on human IL-1β. The locations of receptor activating sites are shown with two downward arrows. (B) Signal peptide sequence is indicated with red color double headed arrow. CXC chemokine family signature is marked with red color box. The location of ELR motif is indicated with double line. CXC domain is indicated within the blue color box. Dimer interface polypeptide binding sites are indicated with upward arrows.

4. Discussion

In the current study, we sought to characterize IL-1β and IL-8 in black rockfish at the molecular level. Moreover, expression levels of these major proinflammatory cytokines were comparatively investigated under pathological conditions to interpret their immunological behavior as inter-related proinflammatory cytokines. Information derived from pairwise sequence comparison and MSA clearly revealed

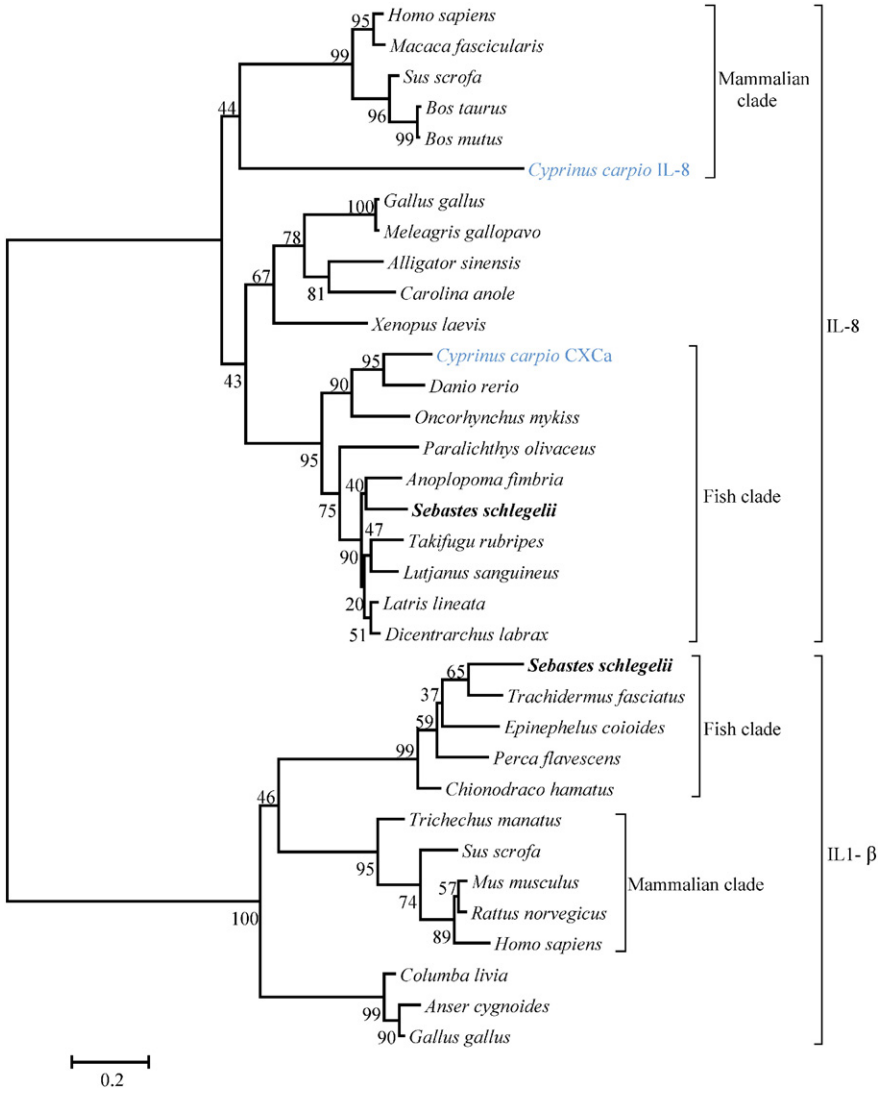

Fig. 2. Phylogenetic analysis of RflL-1β and RflL-8 with selected known full-length IL-1β and IL-8 amino acid sequences of other species. Branch numbers are the bootstrap values for 5000 replicates. Location of rockfish homologs is indicated with bold letters. Two clusters are indicated separately with parenthesis. Corresponding clusters of mammals and fish are indicated within smaller parenthesis. The 2 carp IL-8 counterparts from different lineages are indicated in blue. NCBI-GenBank accession numbers of the amino acid sequences of organisms used in the comparison mentioned in Table 2 except, IL-1β sequences; *Sebastes schlegeli* — KP069025, *Trichechus manatus* — NP_001266185, *Sus scrofa* — CAA52660, *Rattus norvegicus* — AAH91141, *Columba livia* — EMC86541, *Anser cygnoides* — AEL31285 and IL-8 sequences; *Sebaste sschlegeli* — KP069026 *Meleagris gallopavo* — XP_003205721, *Carolina anole* — ENSACAP00000011122, *Macaca fascicularis* — XP_005555144, *Sus scrofa* — NP_999032, *Bos mutus* — XP_005891308, *Cyprinus carpio IL 8* — BAH98111.1, and *C. carpio* CXCa — AJ421443.

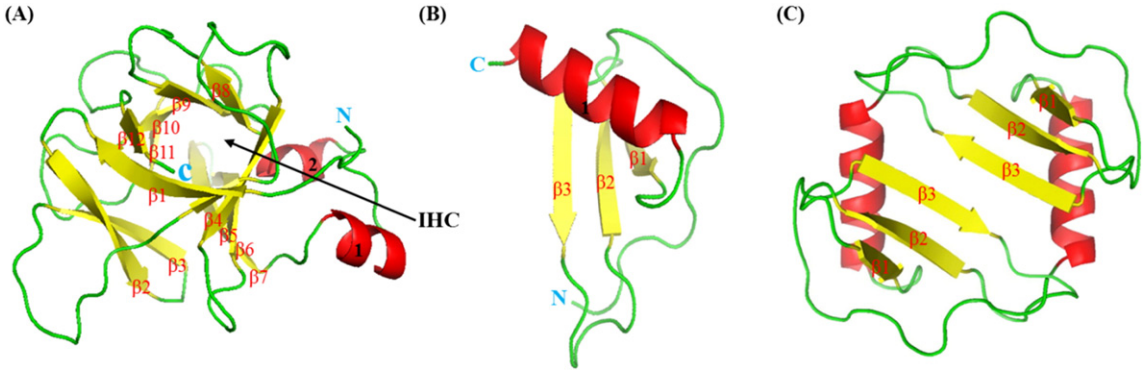

Fig. 3. Tertiary structures of rockfish IL-1β (RflL-1β) and IL-8 (RflL-8) according to the homology based modeling strategy. Carboxyl terminal and amino terminals are indicated with blue color 'C' letter and 'N' letter, respectively. Highly conserved β-strands are colored in yellow and α-helices are colored with red. (A) 3D structure of RflL-1β; interior hydrophobic cavity (IHC) is indicated with arrowhead. (B) 3D structure of RflL-8 monomer.(C) 3D structure of RflL-8 dimer form.

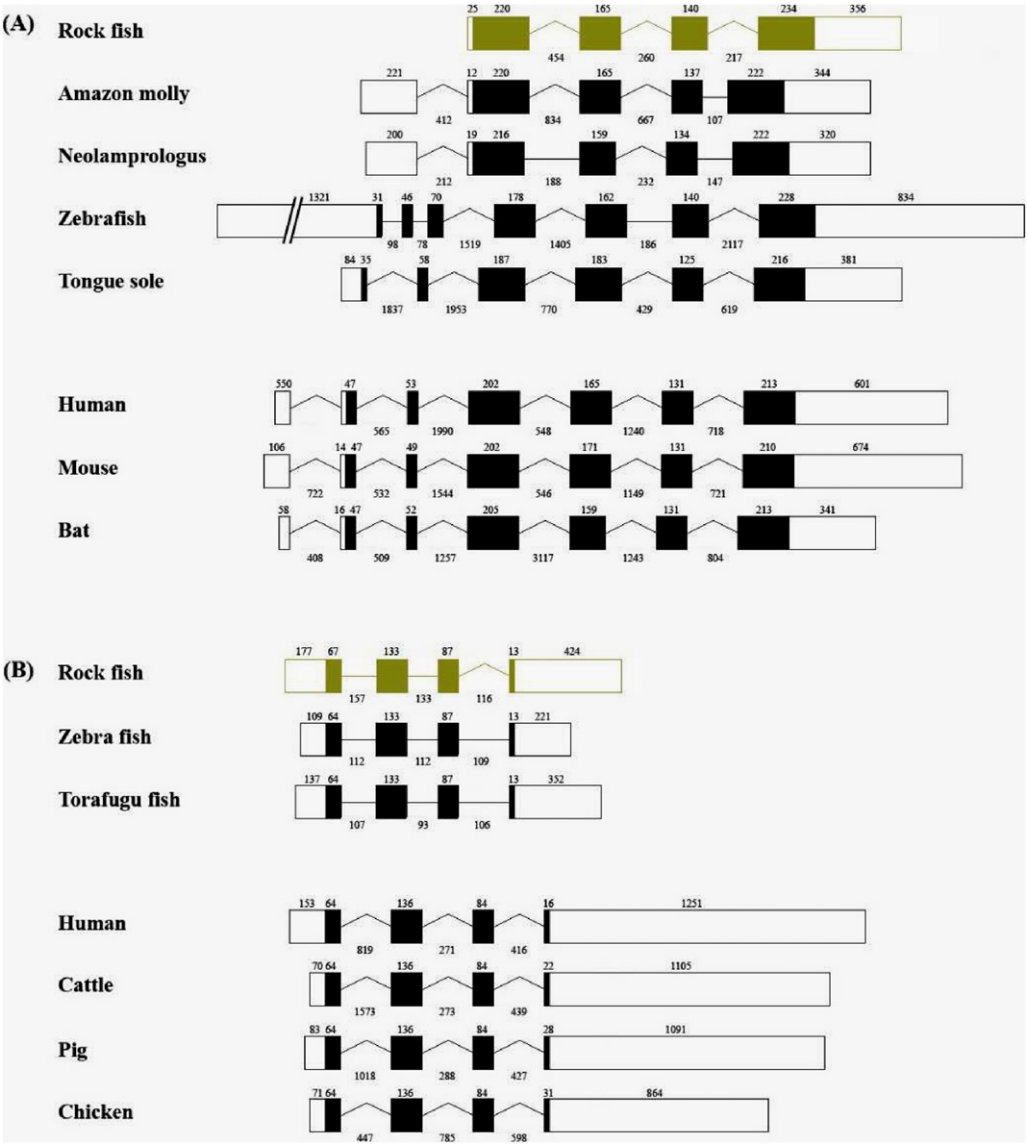

Fig. 4. Genomic organizations of the *IL-1β* (A) and *IL-8* (B) genes from different species. The exons and introns are indicated by boxes and solid lines, respectively. The sizes of exons are indicated above the exons and sizes of introns are indicated below the introns. Black regions represent the coding sequences, whereas white regions represent UTRs. When representing introns, sequence regions larger than 100 bp are truncated by 2 inclined lines. *RflL-1β* and *RflL-8* are indicated in green color. NCBI-GenBank accession numbers for the genomic sequences of organisms used in the comparison are as follows: *IL-1β* sequences; Zebrafish — NC_007121, amazon molly — NW_006800325, neolamprologous — NW_006272053, tongue sole — NC_024320, human — NC_000002, mouse — NC_000068, bat — NW_005370701 and *IL-8* sequences; chicken — NC_006091, pig — NC_010450, human — NC_000004, cattle — AC_000163, zebrafish — NC_007112, torafugu — NW_004072418 and Nile tilapia — NC_022204.

the sequence similarity and identity of aa sequences of black rockfish cytokines with other existing aa sequences from different species (Table 2 and Fig. 1). The high level of sequence similarity among IL-1β sequences (Fig. 1A), especially where the β sheets are gathered, suggests the importance of the integrity of its β-tetroid structure. In addition, previous studies have reported that most of the mammalian IL-1β aa sequences bear a typical D–X (D, aspartic acid residue and X, any hydrophobic residue) as a cutting site for caspase 1 (ICE) in between the prodomain and active domain (Dinarello, 1997). However, RflL-1β does not contain the ICE site which makes it similar to other known teleost IL-1β counterparts. Neither teleost nor avian IL-1β counterparts contain the ICE cleavage site in their aa sequences. It is well known that in mammals, IL-1β is produced as an inactive precursor protein and is cleaved by caspase 1 to generate the mature protein (van der Aa et al., 2010). Further, cleavage sites for caspase 1 are conserved (Dinarello, 1988). Considering previous studies on lower vertebrates, including fish, the notion that teleost IL-1β is produced as a precursor protein and is processed by caspase 1 is still controversial. Other enzymes such as trypsin and chymotrypsin (Hazuda et al., 1990), leukocyte elastase (Black et al., 1988), granzyme A (Irmler et al., 1995), and some bacterial enzymes (Kapur et al., 1993) are also capable of processing the IL-1β precursor. Even if caspase 1 is essential, there may not be a conventional site for precursor processing. According to studies on rainbow trout, IL-1β mature peptide smaller in size than the precursor has been detected with monoclonal antibody mediated strategies, which provides clues for a presence of hypothetical processing mechanism of IL-1β precursor in fish (Hong et al., 2004). It is still unclear whether processing of IL-1β precursor is mandatory for the execution of its function. Extensive studies are necessary to unravel the exact precursor processing mechanisms and to identify cleavage sites and the associated

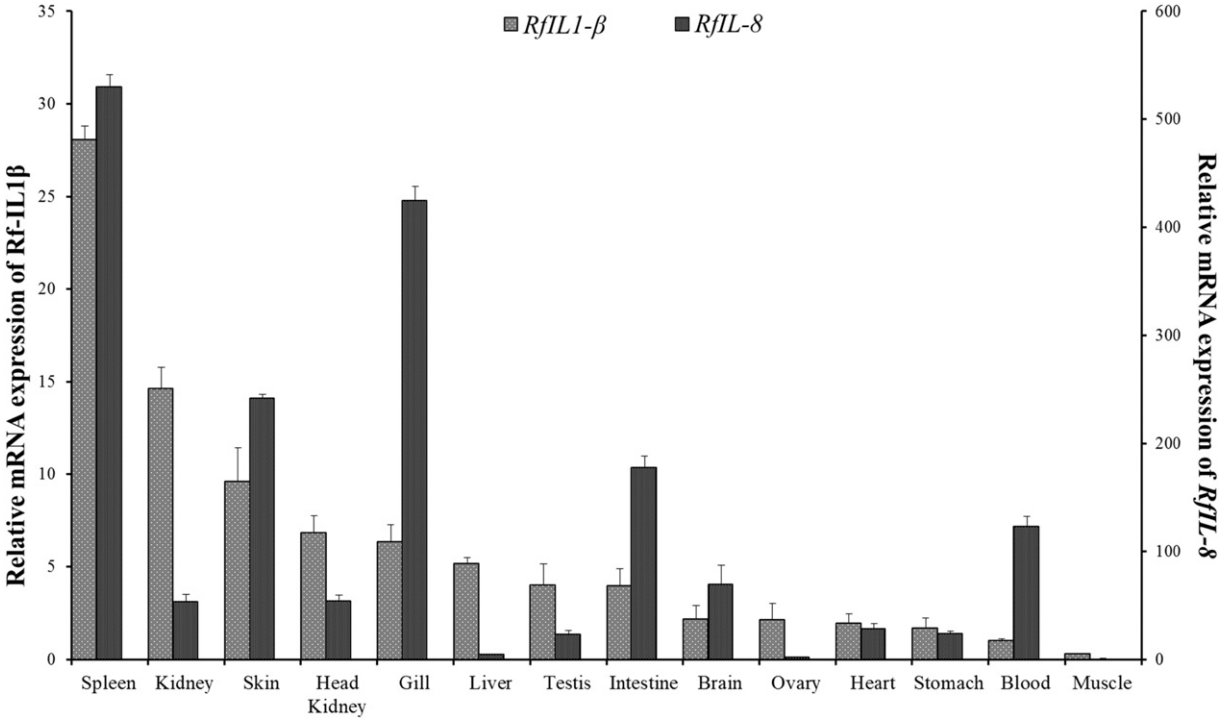

Fig. 5. The tissue specific mRNA expressions of *RfIL-1β* and *RfIL-8*, determined by qPCR. Error bars represent the SD (n = 5). Relative mRNA expression of *RfIL-1β* and *RfIL-8* in different tissues under physiological conditions is represented with primary and secondary axis respectively.

enzymatic counterparts. Detection of signal peptides in the amino acid sequence may predict the potential of a protein to be secreted via the classical pathway, where the Golgi apparatus or endoplasmic reticulum is involved. None of the sequences in black rockfish IL-1β included an N-terminal signal peptide, confirming a non-conventional excretory mechanism (Nielsen et al., 1997).

The presence of a CRC motif in RfIL-8 categorized it as a CXC chemokine (Fig. 1B). Teleostean IL-8 s were distinct from those of other vertebrate in two aspects: (1) containing an Arg in between first two Cys residues, and (2) the ELR motif preceding the CXC signature was absent, except in two species, Atlantic cod (CAD59734) (Seppola et al., 2008) and haddock (AJ566335) (Corripio-Miyar et al., 2007). RfIL-8 possessed an ELH-motif in proximity of it CXC signature. Our phylogeny confirmed the close relationship of RfIL-1β counterpart to that of *Trachidermus fasciatus* and IL-8 counterpart to that of *Anoplopoma fimbria* suggesting their evolutionary relationship with other fish counterparts as well as its deviation from evolutionarily distant species in the phylogenetic chronicle (Fig. 2). In addition, previous studies carried out with teleosts such as carp, revealed 2 IL-8 lineages: one is closely related to the mammalian homolog, whereas the other is likely to be teleost-specific (Abdelkhalek et al., 2009). These may be formed due to a chromosome duplication event during teleost ancestral evolution (Ravi and Venkatesh, 2008). When comparing the evolutionary relationship of our putative RfIL-8 counterpart with orthologs from different carp IL-8 lineages, our RfIL-8 was grouped with a previously identified teleost-specific IL-8 counterpart (*C. carpio* CXCa) along with other fish IL-8 counterparts. This observation further suggests that our putative RfIL-8 counterpart is teleost-specific.

To gain insights into the functional natures of RfIL-1β and RfIL-8 based on their three dimensional (3D) structures, computer based 3D models were generated through homology-based modeling by using the SWISS-MODEL server. RfIL-1β showed a typical β-trefoil structure, an IL-1 family signature (Fig. 3A). Structurally, the β-trefoil structure consists of 12–14 β-strands and 1–2 α-helixes (Finzel et al., 1989; van Oostrum et al., 1991; Yu et al., 1999) with a central hydrophobic cavity. Even though the cavity does not have a direct functional importance, the hydrophobic cavity is likely important for maintaining the structural integrity of the protein (Cheng et al., 2011). In nature, IL-8 exists as a homodimer (Clore et al., 1990). In dimer form (Fig. 3C), two α-helices of each monomers exist in an anti-parallel orientation in order to maintain the structural integrity. The α-helix is known to lay on 3 anti-parallel β-sheet platforms. Specificity of IL-8 receptor binding site is generated due to the distribution of polar residues on α-helical region (Clore et al., 1990). The observed compatibility of predicted structural features with respect to previously annotated mammalian protein structures may suggest similar biological roles for these two interleukin counterparts.

It is likely to have a variable number of exons at the 5′ end of the coding region of IL-1βs, where the prodomain is located, since number of *IL-1β* splice variants have been identified due to incomplete splicing mechanisms in different fish species (Zou et al., 1999). Comparatively, fish species such as tongue sole, zebrafish along with mammalian species compared possessed a high number of exons and introns. According to the intron late theory (Hurst, 1994), the evolutionarily prominent species like mammals might have higher number of introns when compared to fish. Genomic sequences of *IL-1β* of fish species such as zebrafish and tongue sole may have rapidly evolved during speciation as reflected by their intron/exon similarities to mammals (Rogozin et al., 2012). Previous studies on rainbow trout have confirmed the presence of different splice variants, possibly due to incomplete splicing mechanisms, using reverse transcription PCR along with northern and Southern blotting techniques. In the same study, trout *IL-1β* was compared with several mammalian counterparts, reinforcing the outcomes of our genomic comparison, in which the structural arrangement of exons harboring active domains between teleost and mammals were found to be compatible (Zou et al., 1999). With respect to *RfIL-8* genomic sequence (Fig. 4B), the intron-exon arrangement of *RfIL-8* is very similar other examined fish species. In the coding region, the lengths of exons are highly similar to vertebrates suggesting the functional conservation of the *IL-8* gene as an essential chemokine in the animal kingdom. Even though *IL-8* genomic sequence is highly similar among vertebrate species, it is possible to have splice variants due to

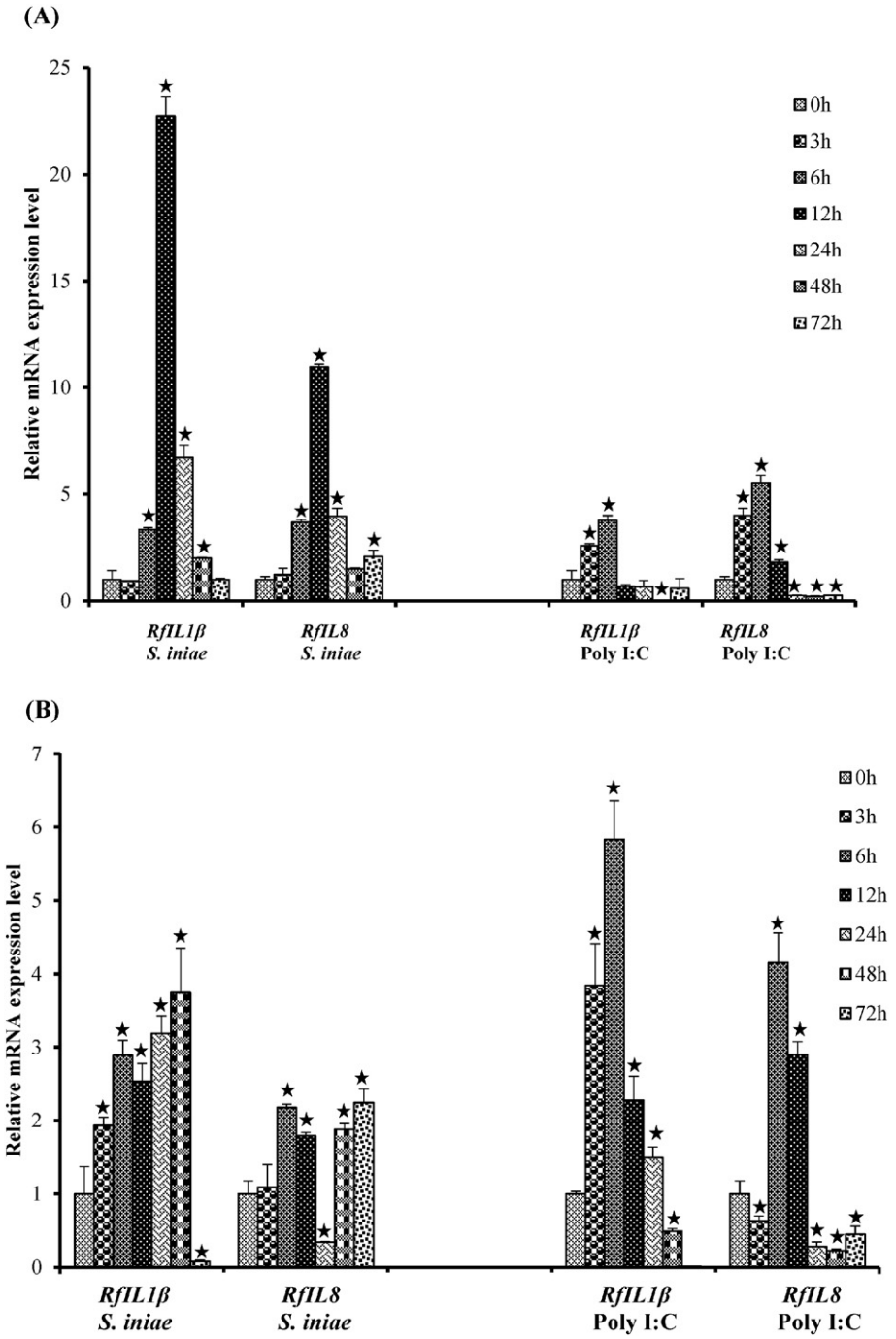

Fig. 6. Transcriptional expression profiles of *RfIL-1β* and *RfIL-8* mRNA in spleen tissue (A) and blood cells (B) upon immune stimulation with *Streptococcus iniae* and poly I:C. The relative expression was calculated by applying the Livak method using *RfEF1A* as a reference gene with respect to corresponding PBS-injected controls at each time point. The relative expression fold change at 0 h post-injection was used as the basal line. Error bars represent the SD (n = 5); and significantly up-regulated and down regulated time points from basal levels are indicated with asterisks ($P < 0.05$).

differences in mRNA processing (Lu et al., 2010) and RNA editing (Li et al., 1999). Extensive studies on the genomic DNA of rainbow trout have identified novel isotypes of rainbow trout IL-8 (Fujiki et al., 2003). Some important residues, such as ELR motif, have undergone modification at aa level in most of the fish species examined (Wang et al., 2013).

In the *RfIL-1β* and *RfIL-8* mRNA expressional profiles, both *RfIL-1β* and *RfIL-8* shared a broad mRNA expressional profile, while exhibiting the highest transcript levels in spleen tissue (Fig. 5). These data suggest a consistent functional importance of *RfIL-1β* and *RfIL-8* in immune relevant organs like spleen (Bronte and Pittet, 2013; MacNeal, 1929), and a broad biological significance in different tissues. In previous studies, relatively similar tissue distribution patterns of these *IL*s were reported in teleosts, such as in orange-spotted grouper (Lu et al., 2008). The pleotropic nature of *IL-1β* expression has also been confirmed in carp (Engelsma et al., 2003; Engelsma et al., 2001), sea bream (Pelegrin et al., 2001), sea bass (Scapigliati et al., 2001) and channel catfish (Wang et al., 2006). A similar tissue distribution pattern of *IL-8* has been detected in catfish (Chen et al., 2005) and Japanese sea perch (Qiu et al., 2009), with significantly higher expression levels in spleen. In contrast, in large yellow croaker, the lowest level of *IL-8* expression was detected in spleen tissue among all tissues examined (Li and Yao,

2013). These data suggest that interleukin mRNA distribution is species-specific.

It is useful to conduct a comprehensive study on mRNA expression levels in immune tissue(s) upon encountering different type of stimuli, in order to prefigure the involvement of pro-inflammatory cytokines such as *IL-1β* and *IL-8* in immunological mechanisms. In this study, in order to examine such putative involvement of *RfIL-1β* and *RfIL-8* in immunological bio-reactions, expression levels of these two *IL* genes were examined under pathologic stresses. Two common potent immune stimuli, *S. iniae*, a Gram-positive infectious bacterium particularly in marine biota, and poly I:C, a viral double stranded RNA mimic were used to trigger the immune responses (Fig. 6). As per inductions seen in spleen tissue challenged with immune stimuli (Fig. 6A), a similar transcriptional behavior of *IL1β* upon bacterial- and/or LPS-challenge has been reported in channel catfish (Wang et al., 2006), sea bream (Pelegrin et al., 2001), roughskin sculpin (Liu et al., 2012) and Atlantic cod (Seppola et al., 2008). Similarly, comparable mRNA expressional profiles of *IL-8* have also been reported in yellow croaker (Li and Yao, 2013) and Japanese sea perch (Qiu et al., 2009) upon bacterial- and/or LPS-challenges. Although Gram positive bacteria, such as *S. iniae*, are not capable of producing LPS, their extracellular polysaccharides (EPS) have been shown to trigger the induction of pro-inflammatory cytokines (Eyngor et al., 2010). Meanwhile, mRNA expressional response of *IL-1β* against nodavirus in sea bream (Poisa-Beiro et al., 2008) and *IL-8* against poly I:C in large yellow croaker (Li and Yao, 2013), respectively, have been reported to have an analogous profile to those seen in our current study. Early phase transcriptional response of these interleukins clearly correlates with their plausible role in triggering the innate immune activities as pro-inflammatory cytokines. Spleen is an important immune organ (Bronte and Pittet, 2013), where phagocytic macrophages reside. IL-1β is an important pro-inflammatory cytokine which has a direct functional importance on macrophage activation and proliferation under pathological conditions (Bird et al., 2002). In blood cells (Fig. 6B), decreased mRNA levels may be associated with the rapid mRNA turnover or evasion mechanisms orchestrated by *S. iniae* (Milani et al., 2010). A number of studies have been carried out in mammalian blood cells to examine the role of IL-8 in inflammation (Darbonne et al., 1991). As an important pro-inflammatory cytokine, IL-8 mediates the leukocyte migration, particularly the infiltration of neutrophil, towards the site of infection and inflammation. While, the increased *RfIL-8* mRNA signified its putative involvement in infection-induced inflammation, elevated transcripts of *RfIL-1β* suggest that it might be involved in activating the other cytokines including *RfIL-8*. On the other hand, studies in rainbow trout and Atlantic cod have proven that the expression level of *IL-8* has an effect on the expression of *IL-1β* (Montero et al., 2008; Seppola et al., 2008). These results may open avenues for a novel study area which could possibly investigate the inter connectivity and similarity in expression pattern of pro-inflammatory cytokines, since they share similar kind of immunological activities within the biological systems.

In summary, we have identified two members of pro-inflammatory cytokines (*RfIL-1β* and *RfIL-8*) from black rockfish at molecular genomic levels and their expression levels were investigated in different tissues under physiological- and pathological- conditions. According to genomic gene structures, while *IL-1β* exhibits a divergent nature, *IL-8* appears to be tightly conserved in different vertebrate species. The synchronized mRNA expressional profiles of these cytokines suggest their inter-related biological function(s). Altogether, present findings of this study suggest the putative involvement of *RfIL-1β* and *RfIL-8* in immunity under inflammatory conditions.

Acknowledgments

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