A THESIS

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Comparative characterization of two interleukin-6 family members in redlip mullet (*Liza haematocheila*) and insights to their functions in regulating inflammation and apoptosis through STAT3 signaling during pathogenic invasion

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Dedication

This study is wholeheartedly dedicated to my beloved teachers, who have been our source of inspiration and gave us strength when we thought of giving up, who continually provide their moral, spiritual, emotional, and financial support

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Summary

The immune system is pivotal in protecting the organism from the exogenous pathogens and other damage associated reactions and molecules. Interleukins are a subgroup of cytokines that can modulate the immune system and involves in critical biological processes. The interleukin 6 family or IL-6 family, in short, has a number of key players in inflammation and other immune responses. IL-6, IL-11, IL-27, IL-31, Leptin, Leukemia Inhibitory Factor (LIF), Cardiotrophin-1 and Ciliary neurotrophic factor (CNTF) are among them. IL-6 acts as anti-inflammatory cytokine through soluble IL-6R receptors. Moreover, IL-6 is serving as a pro-inflammatory cytokine with trans-signaling, through gp130. IL-11 continues as anti-inflammatory and anti-apoptotic cytokine during the inflammation.

In the view of signal transduction accomplishes by both IL-6 and IL-11, they can bind to the specific transmembrane receptor, IL-6 or IL-11 receptor alpha denoted as IL-6Rα or IL-11Rα. Thereby they can activate the JAK-STAT3 pathway. IL6/ IL-6Rα or IL-11/IL-11Rα heterodimers trigger the activation of gp130 in the canonical signaling cascade. The complex of IL protein/ its corresponding receptor and gp130 protein further activates JACK family tyrosine kinases. This catalyzes phosphorylation and translocation of STAT3. The translocated STAT3 can activate transcription of target genes.

The current study was conducted to characterize teleostean IL-6 and IL-11 orthologues from redlip mullet and determine their functional role in mullet kidney cells. Online bioinformatics tools and databases were used together with software to identify characteristic features of the sequences of redlip mullet IL-6 and IL-11. Expression levels of both IL-6 and IL-11 in different tissues of redlip mullet were examined. Groups of healthy redlip mullets were challenged with lipopolysaccharide (LPS), *Lactococcus garviae* and polyinosinic-polycytidylic acid (poly I:C). mRNA was extracted, synthesized cDNA was subjected to quantitative real-time PCR in order to determine expression levels of both interleukins under

immune stimulants. Recombinant IL-6 or IL-11 proteins was overexpressed in *Escherichia coli* BL21 strain and purified using pMAL™ protein fusion and purification system. The purified IL-6 or IL-11 proteins were treated to mullet kidney cells in separate experiments with MBP control. LPS was used to induce inflammation in the cells. Cells were harvested in 0, 1, 3, 6 and 12-hour time points and subjected to mRNA extraction. First strand cDNA was synthesized and qPCR was employed to determine the TNF-α, IL-1β, BCL2 and BAX gene transcription levels in mullet kidney cells during the experiment. Furthermore, the recombinant two interleukins were treated to murine macrophage RAW 264.7 cells to investigate the effect on nitric oxide production.

The redlip mullet IL-6 and IL-11 are composed with 218 and 200 of amino acids respectively. Their predicted molecular weights were 24.6 kDa and 23.168 kDa respectively. The Multiple sequence alignment indicates that both sequences are barely conserved during the evolution. Both of IL-6 and IL-11 of redlip mullet composed with four bundle architecture of α-helixes, as other corresponding orthologues. Identity–similarity matrix demonstrated that a higher identity with fish counterparts but less with other phyla.

Phylogenetic analysis demonstrated that IL-6 and IL-11 have clustered with their fish counterparts respectively. This is the first attempt to clone and characterize both recombinant IL-6 and IL-11 from redlip mullet *Liza haematocheila*. The treatment of recombinant protein into mullet kidney cells has demonstrated that recombinant IL-6 involves in upregulating pro-inflammatory TNF-α, IL-1β significantly. Recombinant IL-11 protein can downregulate the pro-inflammatory TNF-α, IL-1β, and apoptotic BCL2, BAX gene expression in mullet kidney cells. Furthermore, the effect of overexpression of IL-11on STAT-3 signaling in mullet kidney cells was investigated. The experiment revealed that a high dose of IL-11 negatively effects on the transcription of STAT-3 signaling components such as IL-11R, STAT3. However, the transcription of the SOCS3 gene also downregulated by the IL-11 treatment. Additionally, the

results of the current study revealed recombinant IL-6 can induce the inflammatory NO production in Murine RAW 264.7 cells in-vitro, while recombinant IL-11 can reduce the NO production. The overexpression of IL-11 may significantly change the conditions of mullet kidney cells and RAW264.7 cells. Therefore the cells may change the transcription of downstream genes to make a balance in the next stage.

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1. Introduction

1.1 Cytokines, Interleukins, and IL-6 family

Interleukins (ILs) are members of a cytokine family that are used in communication within cells (Hapel and McColl, 1996). Cytokines are highly localized, short-lived, small molecular weight, soluble proteins which are important in autocrine, paracrine and endocrine signaling to coordinates functional activities of the immune system (Rose-John, 2018). Cytokines are categorized into several deferent families according to the type of the receptor. The major cytokine families are, Type I, helical cytokine family or hematopoietin family, Type II or IFN superfamily, Receptor tyrosine –kinase class III ligand family, non-receptor tyrosine –kinase family, tumor necrosis factor family, chemokine superfamily and growth factors with other glycoprotein hormones. Type I or hematopoietin receptor family is the largest family which includes IL-2 family, IL-3 family, IL-6 family, IL-12 family and prolactin family. (Morán et al., 2013).

Interleukine 6 family family includes IL-6, IL-11, IL-27, IL-31, Oncostatin M (OSM) Leukemia inhibitory factor (LIF), Ciliary neurotrophic factor (CNTF), Cardiotrophin-1 (CT-1), Novel neurotrophin-1 (NNT-1) and B cell stimulating factor -3 (BSF-3). All members of this cytokine family are defined by the usage of common β-receptor signaling subunit or gp130 which activate various intracellular signaling pathways (Bauer et al., 2007; Jones and Jenkins, 2018).

1.2 Interleukin 6

The key member of the IL-6 family, IL-6 is a pleiotropic cytokine which has differentiation, growth promoting and inflammatory effects on cells. IL-6 has also been considered to have both pro and anti-inflammatory functions either acutely or chronically

(Northemann et al., 1989). IL-6 is the most common synonym which also referred as B cell differentiation factor (BCDF), B cell stimulatory factor 2 (BSF-2), interferon-β_2 (IFN-β_2) or hepatocyte-stimulating factor (Feghali and Wright, 1997). The first identification, functional characterization, and nomenclature of human IL-6 were suggested in the mid-eighties (Kishimoto et al., 1984; Hirano et al., 1986; Poupart et al., 1987). The first teleostan IL-6 homolog was characterized in 2005 in Japanese pufferfish (*Fugu rubripes*) suggesting the importance of local inflammation (Bird et al., 2005). The second report on the characterization of fish IL-6 concluded that the IL-6 has important functions in the kidney and spleen of Japanese flounder (*Paralichthys olivaceus*) after infection of *Edwardsiella tarda* (Nam et al., 2007).

Basically, IL-6 has been recognized as a pro-inflammatory cytokine. Macrophages, monocytes, and endothelial cells are involved in the production of IL-6 during the acute phase of inflammation. This leads to the activation of a subset of chemokine and other adhesion molecules (Choy and Rose-John, 2017). Furthermore, IL-6 can dictate leucocyte recruitment during the inflammatory response through selective regulation of inflammatory cytokines and apoptosis of cells (Fielding et al., 2014). It can promote the development of Th17 cells with a combination of TGFβ (Kimura and Kishimoto, 2010). On the other hand, it can inhibit TGFβ induced Treg differentiation (Mangan et al., 2006). IL-6 is playing a role as a mediator in neutrophil and macrophage recruitment in the chronic inflammation, thereby pathogenesis of chronic inflammatory diseases (Kumari et al., 2016). Overall summation of IL-6 is a very important cytokine in the point of immunity even disregarding other functions.

1.3 Interleukin 11

Interleukin 11 (IL-11) also subsequently categorized as a member of the interleukin-6 (IL-6) family of cytokines is another important secreted cytokine in the IL-6 family. It possesses pleiotropic and diverse functions in many tissues and cells (Dams-Kozlowska et al.,

2012). The first observation of IL-11 was made from interleukin-6 dependent primate bone marrow-derived PU-34 cell line as a hematopoietic cytokine. As a member of the IL-6 family, IL-11 plays important hematopoietic roles in the production of thrombocytes and megakaryocytes, bone formation, cell differentiation, and placental development (Santos et al., 2008). Moreover, IL-11 has also shown notable anti-inflammatory activities by suppressing the expression of pro-inflammatory cytokines and by inhibiting the formation of nitric oxide (NO) (Harmegnies et al., 2003; Schwertschlag et al., 1999).

The first teleostean IL-11 was characterized in rainbow trout (*Oncorhynchus mykiss*), suggesting that IL-11 is an active player of the fish immune response during infections (Wang et al., 2005). Thereafter, two paralogous IL-11 sequences (IL-11a and IL-11b) have been identified in several fish species, such as zebrafish, common carp and tiger pufferfish (Huising et al., 2005). Another study on teleost IL-11 has demonstrated that the IL-11a mRNA was ubiquitously expressed in all tissues except PBLs while the IL-11b mRNA was highly expressed only in PBLs. Both of the IL-11 paralogues in fish appear to be functional and play complementary roles in immune response (Santos et al., 2008).

1.**4 Signal transduction of IL-6 and IL-11**

The receptors involved in recognition of the IL-6 family cytokines can be subdivided into two categories such as non-signaling α-receptors (IL-6Rα, IL-11Rα, and CNTFRα,) and the signal transducing receptors (gp130, LIFR, and OSMR)(Mousa and Bakhiet, 2013).

IL-6 can stimulate the target cell response in two different ways by two types of IL-6/IL-11 receptors. The cells, which only express gp130 are completely unresponsive to IL-6 stimulations (Rose-John, 2012). Membrane-bound IL-6R/IL-11R is involving in classical signaling. The IL-6/IL-11 binds to the membrane-bound IL-6R/IL-11 which initiates dimerization of gp130 (figure 1) (Agthe et al., 2017; Taga et al., 1989). Alternatively, IL-6 can

bind to the soluble IL-6 receptor (sIL-6R) which generates by cleavage of membrane-bound IL-6R by metalloproteinase domain-containing protein -17 (ADAM-17) and Disintegrin. Thereafter the heterodimer of IL-6/sIL-6R or IL-11/sIL11R binds to membrane-bound gp130 in trans-signaling (Figure 1) (Rose-John et al., 2017).

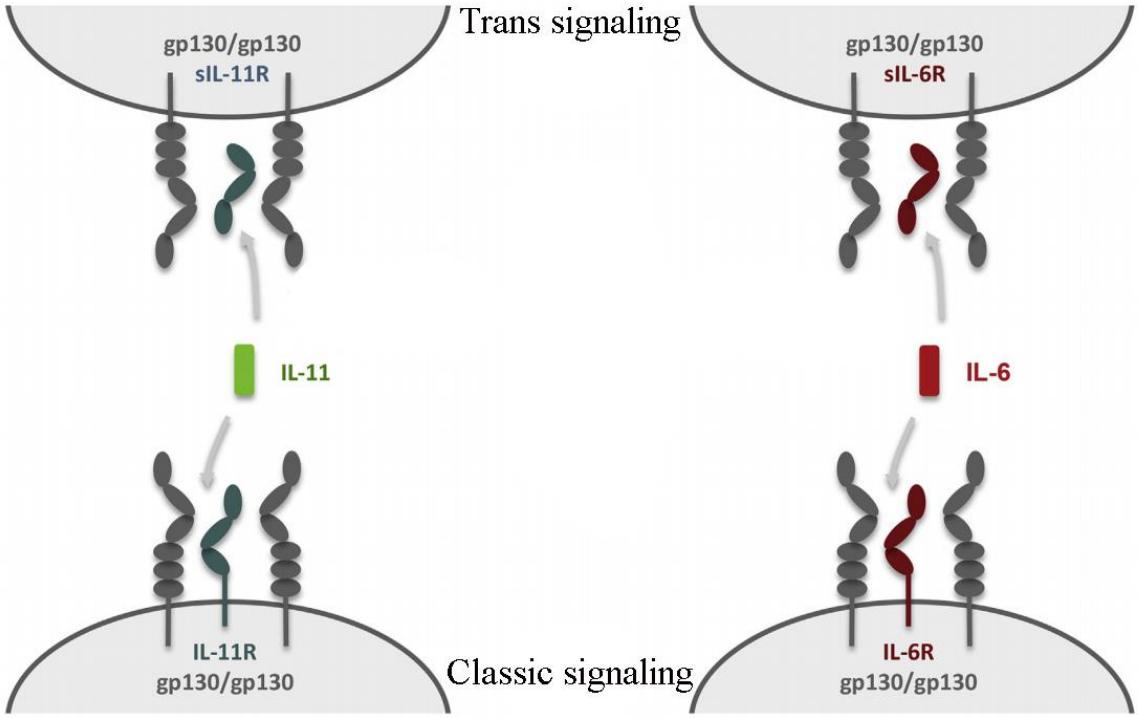


Figure 1. Classic and trans-signaling of IL-6 and IL-11

The activation of gp130 involves the activation of JAK (Janus kinase) tyrosine kinase family members, causing to the activation of transcription factors of the signal transducers and activators of transcription (STAT) family. Especially, IL-6 and IL-11 activate STAT1 and STAT 3 transcription factors (Ernst et al., 2008; Hodge et al., 2005).

STAT proteins including STAT1, STAT3, and STAT-likes are kept in an inactive cytoplasmic form in non-stimulated cells. Once activated, STAT starts the translocation into the nucleus where it acts as a transcription activator for an extensive array of targeted genes. Typically, STAT3 activation is induced by phosphorylation on a critical tyrosine residue (Tyr 705) that triggers STAT3 dimerization due to reciprocal phosphotyrosine-SH2 domain

interactions. Even if multiple tyrosine kinases have been described as intracellular activators of STAT3 activity, the phosphorylation of STAT3 on tyrosine 705 is mainly regulated by members of Janus-activated kinases with JAK1 as a key modulator. In addition to tyrosine 705 phosphorylation, STAT3 has also activated through serine (Ser 727) phosphorylation. This phosphorylation is commonly regulated by protein kinase C, mitogen-activated protein kinases, and CDK5. Finally, the reversible acetylation of STAT3 by histone acetyltransferase on a single lysine residue at the position 685(Lys 685), represents the third mechanism of STAT3 activation. Acetylated STAT3 has been depicted as a way to enhance the stability of STAT3 dimers which are required for DNA-binding and transcriptional activity (Rébé et al., 2013).

1.5 Significance of STAT3 transcription factor in the mediation of inflammation

STAT 3 is having universal and cell type-specific binding ability with a self-regulatory mechanism. Therefore, continues STAT3 transcription may be allowed by binding to its own promoter, acts as a master regulator of important transcription factors downstream cascades, transcribes specific signaling modulators and cytoplasmic enzymes that fine-tune STAT3 signaling and ensures a robust cell division program and the maintenance of a stable phenotype (Hutchins et al., 2013) .

Some of the target genes of STAT3 such as suppressor of cytokine signaling-3 (SOCS3), BCL3 and the protein tyrosine phosphatase Ptpn1, a negative regulator of JAK-STAT signaling that works by dephosphorylating phospho-STAT3 (Yasukawa et al., 2003). The DNA-binding preferences of STAT3 are indistinguishable from those of most other STAT family members, a ‘DNA binding degeneracy’ trait common to virtually all transcription factor families (Jolma et al., 2013).

1.6 Redlip mullet aquaculture in South Korea

Redlip mullet (*L*iza *haematocheila)* is a teleost fish species belonging to the family Mugilidae. It naturally inhabits in all of the water types in the Northwest Pacific and has been recently introduced to the Black Sea and the Mediterranean (Zenetos et al., 2005). Redlip mullet is able to adapt to the changing salinities and grow up to 80 cm in length and 5kg in mass (Kottelat and Freyhof, 2007). Due to these desirable characteristics, it has become an important candidate for aquaculture in China and the Korean peninsula (Han et al., 2015). Nevertheless, redlip mullets in the intensive aquaculture are susceptible to various pathogenic diseases, including vibriosis, streptococcosis, bacterial fin rot, and motile Aeromonas septicemia, which cause high mortalities and lead to huge economic losses (Han et al., 2015). *The s*tudy of immune mechanisms of redlip mullet is crucial for the prevention and treatment of these diseases. In this study, efforts were made to investigate the molecular features of a teleostean IL-11a ortholog from redlip mullet (*Lh*IL-11a) and to evaluate the evolutionary relationships and homology with known IL-11 homologs. The immune response of *Lh*IL-11a mRNA was examined after pathogenic challenge using polyinosinic-polycytidylic acid (thermosensitive poly I:C), lipopolysaccharide (LPS) and *Lactococcus garviae*. The biological activity of *Lh*IL-11a was further elucidated using recombinant *Lh*IL-11a and redlip mullet kidney cells.

2. Material and methods

2.1 Rearing of fish and tissue collection

Healthy redlip mullets with an average body weight of 100 g were purchased from Sangdeok Fishery fish farm in Hadong, Korea. The fish were transported to the laboratory and acclimatized in 300 L aquarium tanks at 20°C for one week prior to experimentation. MS-222(40mg/L) was used to anesthetize experimental fish prior to tissue dissection. Blood was collected using heparin sodium salt (USB, USA) coated sterile syringes, and the peripheral blood cells (PBCs) were separated by immediate centrifugation at 3,000 × g for 10 min at 4°C. Other tissues including kidney, head kidney, spleen, liver, gill, intestine, brain, skin, heart, and stomach were dissected and immediately snap-frozen in liquid nitrogen storage at -80°C.

2.2 Transcriptomic database construction

The transcriptome database of redlip mullet cDNA sequences was constructed by PacBio and Illumine NGS technologies. In brief, five healthy redlip mullets were dissected and total RNA was extracted from eleven tissues. Aliquots of sampled RNA were sent to run sequencing on a Pacbio platform at Insilicogen, Korea. After complete sequencing, the results were *de novo* assembled and annotated using Blast2Go software (https://www.blast2go.com/).

2.3 Bioinformatics analysis

A sequence from the constructed database, with the highest homology with IL-6 and IL-11a, was identified using the Basic Local Alignment Search Tool (BLAST). The open reading frame (ORF), amino acid sequence, theoretical isoelectric point, and molecular mass of the corresponding protein were predicted using the CLC Main Workbench (Version 8.0.1) program and protparam online tool. Putative signal peptides were predicted by SignalP. Characteristic domains and motifs of both of *Lh* IL-6 and LhIL-11a were identified by Motif

Scan. *Lh*IL-6 and *Lh*IL-11a amino acid sequences were aligned with the sequences of other known *Lh*IL-11a and IL-11 genes. The phylogenetic tree was constructed using the Neighbor-Joining method in BioEdit sequence alignment editor (Version 7.2.5) and the CLC Main Workbench (Version 8.0.1). The tertiary structure of both *Lh*IL-6 and *Lh*IL-11a were predicted by Phyre^2 Protein homology/analogy recognition engine version 2.0 and PyMOL Molecular Graphic System (Version 2.0.7), using the crystal structure of human interleukin-6 and 11 as a template.

2.4 Immune challenge experiment

To study the transcriptional modulation of *Lh*IL-6 and *Lh*IL-11a under different pathogenic occurrences, LPS (125 µg/g-fish), poly I:C (150 µg/g-fish) or *Lactococcus garvieae* (1×10^5 CFU/fish) were intraperitoneally injected to healthy redlip mullet in a total volume of 100 µL. The same volume of PBS was injected into the control group fish. The PBCs and liver from five individuals were sampled at 0, 6, 24, 48, and 72 hours post-injection.

2.5 Total RNA isolation and cDNA synthesis

Total RNA was isolated from pooled tissue samples of five individual fish for tissue distribution and immune challenge using the RNAiso plus kit (Takara, Japan). The RNA was purified using RNeasy kit (Qiagen, USA). Agarose gel electrophoresis and a µDrop Plate (Thermo Scientific) were used to examine the RNA quality. First-strand cDNA synthesis was performed in a 20 µL reaction mixture using 2.5 µg of RNA and the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara). The synthesized cDNA was diluted (40X) in nuclease-free water and stored at −80°C.

2.6 Analysis of spatial and temporal IL-6 and IL-11expression variation by real-time PCR (qRT-PCR)

qRT-PCR was employed using a TaKaRa Thermal Cycler Dice™ TP950 (Japan). The total reaction mixture was 10 µl, including 3 µl of diluted cDNA template, 5 µl of 2X TaKaRa

Ex Taq™ SYBR premix, 1 µl of 1:1 mixture of forward and reverse primers (10 pmol/µl) and 1 µl of H_2O. The qRT-PCR cycle profile was composed as follows: one cycle of 95°C for 10 s, 45 cycles of 95°C for 5 s, 58°C for 10 s and 72°C for 20 s, and a last cycle of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Triplication of each assay was performed to increase the credibility. The Livak $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression (Livak and Schmittgen, 2001). Elongation Factor 1 alpha (EF1α; accession no. MH017208) was used as the internal control gene. Data were finally presented as average relative mRNA expression ± standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's *post-hoc* comparison test was used to analyze tissue-specific mRNA expression data. The unpaired Student's *t*-test was used to assess statistical significance in the immune challenge experiment using Minitab® (release 15) software. Statistical significance was assumed when $p < 0.05$.

2.7 Plasmid construction for recombinant *Lh*IL-6 and *Lh*IL-11a production

*Lh*IL-6 and *Lh*IL-11a coding sequence were PCR amplified using sequence-specific primers (Table 1) and sub-cloned into the pMAL-c5X vector following the provided protocol (New England Biolabs, Ipswich, MA, USA). Briefly, PCR amplified products were purified with PCR purification kit (AccuPrep® PCR Purification Kit, BIONEER, South Korea). The purified products were simultaneously digested with *pMAL*-c5X vector with the corresponding restriction enzyme (Table 1) at 37ºC for three hours. Digested fragments were isolated using agarose gel electrophoresis followed by gel purification (AccuPrep® Gel Purification Kit, BIONEER, South Korea). Digested products were ligated using Mighty Mix DNA Ligation Kit (TaKaRa, Japan). Then, the recombinant plasmids were transformed into *Escherichia coli* competent cells (DH5α). Transformed DH5α cells were grown on Luria-Bertani (LB) plate containing ampicillin at 37 °C overnight. Successive clones were recognized and the sequence

was verified by sequencing (Macrogen, Korea). Sequence confirmed plasmids were transformed into *E. coli* BL21 competent cells for expression of *Lh*IL-6 and *Lh*IL-11a.

Table 1 Description of primers

Primer name	Primer sequence
*Lh*IL-6 -qPCR Forward	5'-CGAGCGCCACAAGAAGAAGTTTGA-3'
*Lh*IL-6 -qPCR Reverse	5'-GAGACAAGCCTCCTTGCTGAAGTTG-3'
*Lh*IL-6 -Cloning Forward	5'-gagagagtcgacATGCCCTCTAAAATTAACTTGGTG-3'
*Lh*IL-6 -Cloning Reverse	5'-gagagaggatccTTAAGCACTGTGCCATATTCCTGTC-3'
*Lh*IL-11a -qPCR Forward	5'-CCACAGGATCTGGCGCATGT-3'
*Lh*IL-11a -qPCR Reverse	5'-CCTATCAGTTTGATGTCCTCCAGTCCAG-3'
*Lh*IL-11a -Cloning Forward	5'-gagagaggatccATGAAATTGCTGCTCGACTCCTC-3'
*Lh*IL-11a -Cloning Reverse	5'-gagagagaattcTCATTGTACTGCAGACACTTTGGG-3'
*Lh*TNFα-qPCR Forward	5'-CCACAGGATCTGGCGCATGT-3'
*Lh*TNFα -qPCR Reverse	5'-CGAGGTAGATGGCGTTGTACCAG-3'
*Lh*IL-1β-qPCR Forward	5'-GGGCATCAAGGACACAGACCTCTA-3'
*Lh*IL-1β -qPCR Reverse	5'-GTCGCGCTTGTAGAAGAGAAACCG-3'
*Lh*BCL2-qPCR Forward	5'-TCGAGTGCGTGGAGAAGGAGATG-3'
*Lh*BCL2-qPCR Reverse	5'-GTCCTGCCCGAAGATTTCAGCAAAG -3'
*Lh*BAX -qPCR Forward	5'-CGGGATCACGGAGGATGGATCAATAG-3'
*Lh*BAX - qPCR Reverse	5'-TAGAGTCACGCCAGCGATGAACA-3'
*Lh*STAT3- qPCR Forward	5'-GCTGAGGTGTTGAGCTGGCAAT-3'
*Lh*STAT3- qPCR Reverse	5'-AGAAGCCTTTGCCAGCCATGTT-3'
*Lh*IL11R- qPCR Forward	5'-ATCCTGCATTCTGGCAGACCTT -3'
*Lh*STAT3- qPCR Reverse	5'-TTCCATGACACGACCAGCTTCT -3'
*Lh*SOCS3- qPCR Forward	5'-CACCACACTTCACAAGCTCCAGG-3'
Lh SOCS3- qPCR Reverse	5'-CACCACACTTCACAAGCTCCAGG-3'

2.8 Overexpression, and purification of *rLh*IL-6 and *rLh*IL-11a

The recombinant *Lh*IL-6 and *Lh*IL-11a protein was overexpressed and purified according to the manufacturers manual. Briefly, recombinant *Lh*IL-6 and *Lh*IL-11a proteins were overexpressed as a fusion protein with a maltose binding protein tag in *E. coli* BL21 cells. Transformed *E. coli* BL21 cells were grown in LB rich ampicillin medium (500 mL) which contained 100 mg mL^{-1} ampicillin and 0.2% glucose. The culture was incubated at 37 °C and 200 rpm in a shaking incubator. After 24 hour incubation period, the seed culture was inoculated into LB rich medium containing ampicillin (50μg/mL) and incubated at 37 °C and

200 rpm until the OD_{600} reached 0.5. Isopropyl-b-thiogalactopyranoside (IPTG; 0.25 mM) was added into the culture medium to induce the expression of the protein at 20 °C and 200 rpm for 10 h. The cells were harvested at 4500 x g for 30 min at 4 °C. Harvested cells were re-suspended in the column buffer, which contained 20 mM Tris-HCl, 200 mM NaCl, pH 7.4, and stored at -20 °C.

The bacterial suspension was thawed and 1 mg mL^{-1} of lysozyme was added for cell lysis. Thereafter, the lysed cells were sonicated and the cell lysate was centrifuged at 20,000 x *g* at 4 °C for 30 min. The supernatant was separated and purified on a column with amylose resin (New England Biolabs, USA). The purified r*Lh*IL-6 and r*Lh*IL-11a were eluted using elution buffer composed of column buffer and 10 mM maltose. Simultaneously, overexpression and purification of the recombinant MBP (rMBP) were completed. Concentrations of purified r*Lh*IL-6 and r*Lh*IL-11a and rMBP were measured using the Bradford method (Bradford, 1976). Efficacy of protein purification was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE) under reducing conditions.

2.8 *rLh*IL-11a treatment in mullet kidney cells

2.8.1 Primary culture of mullet kidney cells

The kidney tissues were dissected from healthy redlip mullets. After three washes with PBS, the tissues were transferred to a sterile dish including L-15 medium (Sigma, USA) supplemented with 40% fetal bovine serum (FBS; Hyclone), 100 μL/mL penicillin and 100 μg/mL streptomycin (1X P/S) (Gibco). The kidney tissues were shredded and filtered through a 40 μm cell strainer (SPL). Filtered single cells were seeded in a 6-well cell culture plate and incubated at 25℃. The medium was changed to L-15 medium supplemented with 10% FBS

and 1X P/S after 7 days. The cells were subcultured when the cell populations were stably proliferated. All experiments were performed within 20 passages.

2.8.2 *rLh*IL-6 or *rLh*IL-11a treatment and qRT-PCR for pro-inflammatory and apoptotic gene expression.

Mullet kidney cells were seeded in two 12-well cell culture plates and incubated for two days at 25℃. Different amounts of *rLh*IL-6 and maltose binding proteins (MBP) proteins were added to the media to make final concentration 0.5, 1.0, 10.0 ng/mL and 10.0 ng/mL respectively. Cells were incubated at 25^{o}C and harvested at 0, 1, 3, 6, and 12 hour time points and stored -80^{o}C.

In another experiment, mullet kidney cells were seeded in two 12-well cell culture plates and incubated for two days at 25℃. *rLh*IL-11 and maltose binding protein (MBP) proteins were added to the medium of one plate each at a final concentration of 100 ng/mL. Half of the wells on each plate were treated with LPS (2 μg/mL). The treatments were denoted as IL-11 (+LPS), IL-11 (-LPS), MBP (+LPS), and MBP (-LPS). Cells were harvested at 0, 1, 3, 6, and 12 hours post-treatment and stored -80^{o}C.

RNA extraction and cDNA synthesis were performed for both experiments as described above. qPCR was performed for four redlip mullet genes associated with inflammation and apoptosis (*Lh*IL-1β, *Lh*TNFα, *Lh*BCL2, and *Lh*BAX) using the primers listed in Table 1. Furthermore, the feedback transcriptional response of STAT3 signaling cascade components was evaluated using qPCR using the primers listed in Table 1. Data were analyzed as described in section 2.6.

2.9 NO production assay and MTT assay

To determine the effects of *rLh*IL-6 or *rLh*IL-11a on nitric oxide (NO) production, murine macrophage (RAW264.7) cells were treated with recombinant proteins and monitored for NO production. In brief, RAW264.7 cells were seeded into 24-well cell culture plates in complete Dulbecco's Modified Eagle's Medium (DMEM) at 37°C. After reaching to 80-90% confluence the cells were treated with MBP (10 ng/mL), *rLh*IL-6 or *rLh*IL-11a (1, 5, 10 ng/mL) with heat-denatured proteins (10 ng/mL) for one hour. NO production was then stimulated with 100 ng/mL LPS treatments in the *rLh*IL-11a experiment. After further 24-h incubation, the supernatant from each well was transferred to a 96-well plate and incubated with Griess reagent (1% sulfanilamide and 0.1 naphthylenediamine dihydrochloride in 197 2.5% of phosphoric acid) for 10 minutes at room temperature, for color development. Absorbance was measured at 540 nm in a microplate reader (Multiskan GO, Thermo Scientific, USA). A cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay; MTT) was conducted to evaluate the cell survival rate during the experiment in the presence of *rLh*IL-6 or *rLh*IL-11a. The cell viability of the above experiment was measured using the microplate reader (Multiskan GO, Thermo Scientific, Finland) at an absorbance of 540 nm. Each experiment was performed in triplicate. MBP (+LPS) treated cells were considered to have 100% NO production. Statistical significance was analyzed by one-way ANOVA and the correlation between *rLh*IL-11a and NO production was analyzed by correlation test using Minitab® (release 15).

3. Results and Discussion

3.1 *Lh*IL-11a sequence analysis

The complete ORFs (Figure 2) of *Lh*IL-6 and *Lh*IL-11a are 657 bp and 603 bp in length. The deduced amino acid sequence contains218 and 200 amino acids with a predicted molecular weight of 24.6kDa and 23.16 kDa respectively. The theoretical isoelectric points were 8.38 and 9.16 respectively. In both amino acid sequences, 25aa and 26aa long signal peptides were predicted.

(A)

(B)

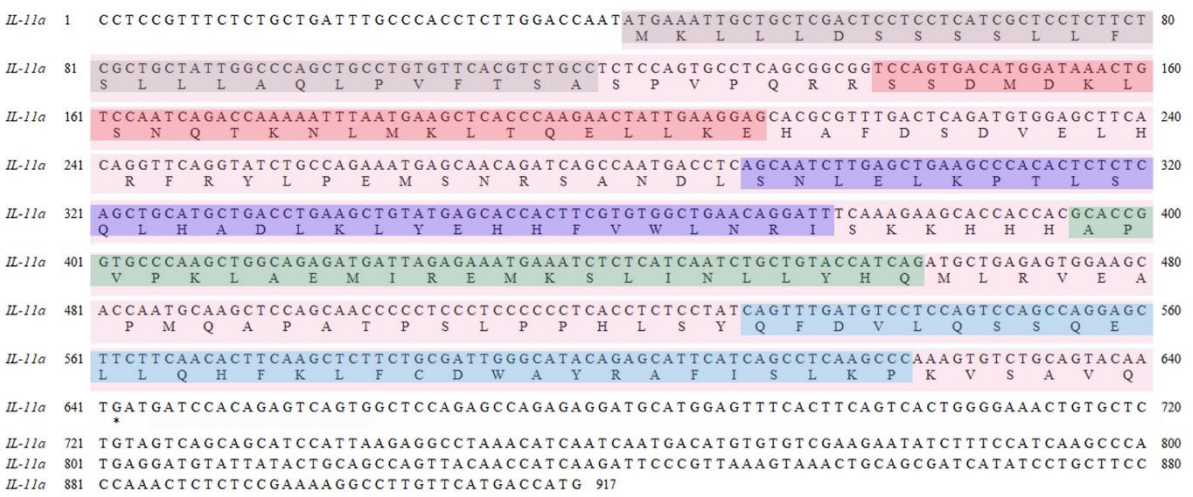


Figure 2. Sequence analysis figures of *Lh*IL-6 and *Lh*IL-11a

Sequence analysis figures of *Lh*IL-6 (A) and *Lh*IL-11a (B). Open reading frames (ORFs) are marked in pink, predicted signal peptides are marked in ash color, α helix A's are marked in red, α helix B's are marked in purple, α helix C's are marked in green, α helix D's are marked in blue.

3.2 Homology analysis of *Lh*IL-6 and *Lh*IL-11a

Protein-protein BLAST of both amino acid sequences in the SwissProt database shows significant hits with the IL-6 and IL-11 orthologs from teleost species respectively. *Lh*IL-6 shares the highest identity (46.7%) and similarity (61.4%) with *Tetraodon nigroviridis*_IL-6. *Lh*IL-11a shares the highest identity (84.6%) and similarity (91.5%) with *Takifugu rubripes* IL-11a. The identity-similarity table (Table 2 (B)) shows that *Lh*IL-11a has a significantly higher identity (46.6 – 84.6%) with fish IL-11a sequences than with fish IL-11b sequences (27.1– 33.8%).

Table 2. Identity –similarity of *Lh*IL-6 (A) and *Lh*IL-11a (B)

(A) Identity –similarity of *Lh*IL-6

	Organism (IL-6)	Taxonomy	Identity (%)	Similarity (%)
1	*Epinephelus coioides*	Teleost	46.7	61.4
2	*Larimichthys crocea*	Teleost	37.9	59.1
3	*Oreochromis niloticus*	Teleost	46.7	63.6
4	*Paralichthys olivaceus*	Teleost	45.7	60.9
5	*Hippoglossus hippoglossus*	Teleost	45.2	62.4
6	*Xenopus tropicalis*	Amphibia	16.3	35.8
7	*Python bivittatus*	Reptilian	21.5	41.3
8	*Gallus gallus*	Aves	22.4	42.3
9	*Canis lupus*	Mammalia	19.5	42.7
10	*Sus scrofa*	Mammalia	21	42.2
11	*Homo sapiens*	Mammalia	18.6	40.8

(B) Identity –similarity of *Lh*IL-11a

	Organism (IL-11)	Taxonomy	Identity (%)	Similarity (%)
1	*Tetraodon nigroviridis*_IL11a	Teleost	84.6	91.5
2	*Takifugu rubripes*_IL11a	Teleost	82.5	92.5
3	*Liza haematocheila*_IL11	Teleost	61.5	81.4
4	*Oncorhynchus mykiss*_IL11	Teleost	57.7	79
5	*Carassius auratus*_IL11	Teleost	56.7	79
6	*Cyprinus carpio*_IL11	Teleost	46.6	68
7	*Danio rerio*_IL11a	Teleost	33.8	56.7
8	*Tetraodon nigroviridis*_IL11b	Teleost	29.7	55.4
9	*Takifugu rubripes*_IL11b	Teleost	29.6	53.7
10	*Hippoglossus hippoglossus*_IL11b	Teleost	29.1	53.5
11	*Paralichthys olivaceus*_IL11b	Teleost	27.5	51
12	*Danio rerio*_IL11b	Teleost	27	47
13	*Sus scrofa*_IL11	Mammalia	26.4	47.5
14	*Homo sapiens*_IL11	Mammalia	26.4	44.5
15	*Mus musculus*_IL11	Mammalia	27.1	52.9

3.3 Sequence alignment of *Lh*IL-6 and *Lh*IL-11a with other known homologs.

There is one conserved cysteine residue (C^{95}) in the interloop between αhelix A - αhelix B and another one (C^{105}) in α helix B of *Lh*IL-6 peptide sequence. One conserved cysteine residue (C^{183}), near the C-terminus of the fish IL-11 peptide sequence, is considered important for the stability of protein structure (Figure. 3) (Huising et al., 2005). However, there is no cysteine found in the IL-11 sequences of mammals. The lack of this cysteine might be compensated by the hydrophobic interactions of the abundant leucines and the amino acids with a hydrophobic side chain, such as alanine, proline, and tryptophan, which are highly conserved especially in α-helixes (Czupryn et al., 1995).

αhelix B and αhelix D of both proteins are more conserved in comparison with the other two αhelixes. The receptor binding sequence is in the αhelix D, therefore, the sequences would be conserved during the evolution.

(A)

(B)

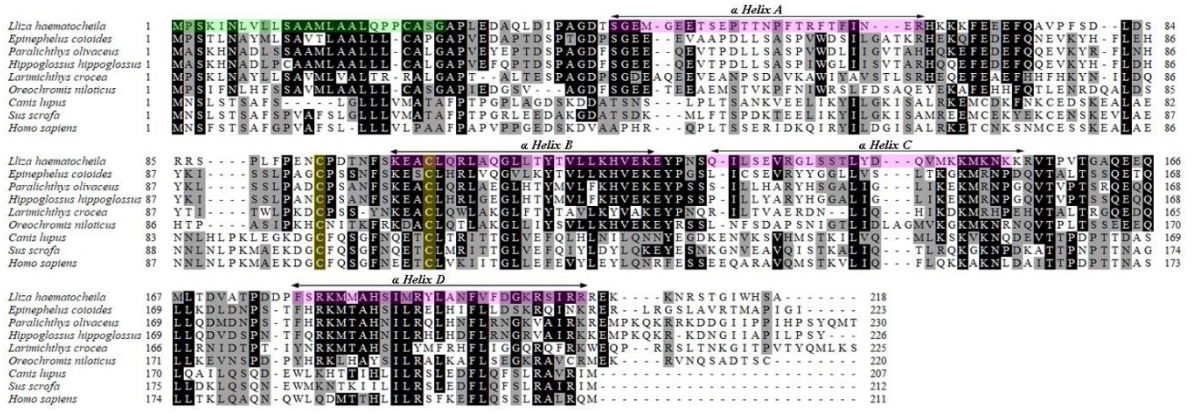


Figure 3. Multiple sequence alignments of *Lh*IL-6 and *Lh*IL-11a

Multiple sequence alignment of the deduced *Lh*IL-6 **(A)**, *Lh*IL-11a **(B)** amino acid sequence with other IL-6 and IL-11a homologs. The predicted signal peptide marked in green, four α helices are marked in purple. Conserved cysteine residues are highlighted in yellow.

3.4 phylogenetic relationship reconstruction of *Lh*IL-6 and *Lh*IL-11a

The phylogenetic tree reconstructed with *Lh*IL-11a and other IL-11 homologs of different phyla was rooted with another family member, IL-6 (Figure. 4). Two distinct clades were observed in the fish IL-11 branch with a 72% bootstrap value. *Lh*IL-11a was clustered within the clade containing IL-11a and unclassified IL-11 genes whereas the other clade was composed solely of IL-11b sequence. This result confirms that *Lh*IL-11a is an ortholog of other teleostean IL-11a sequences.

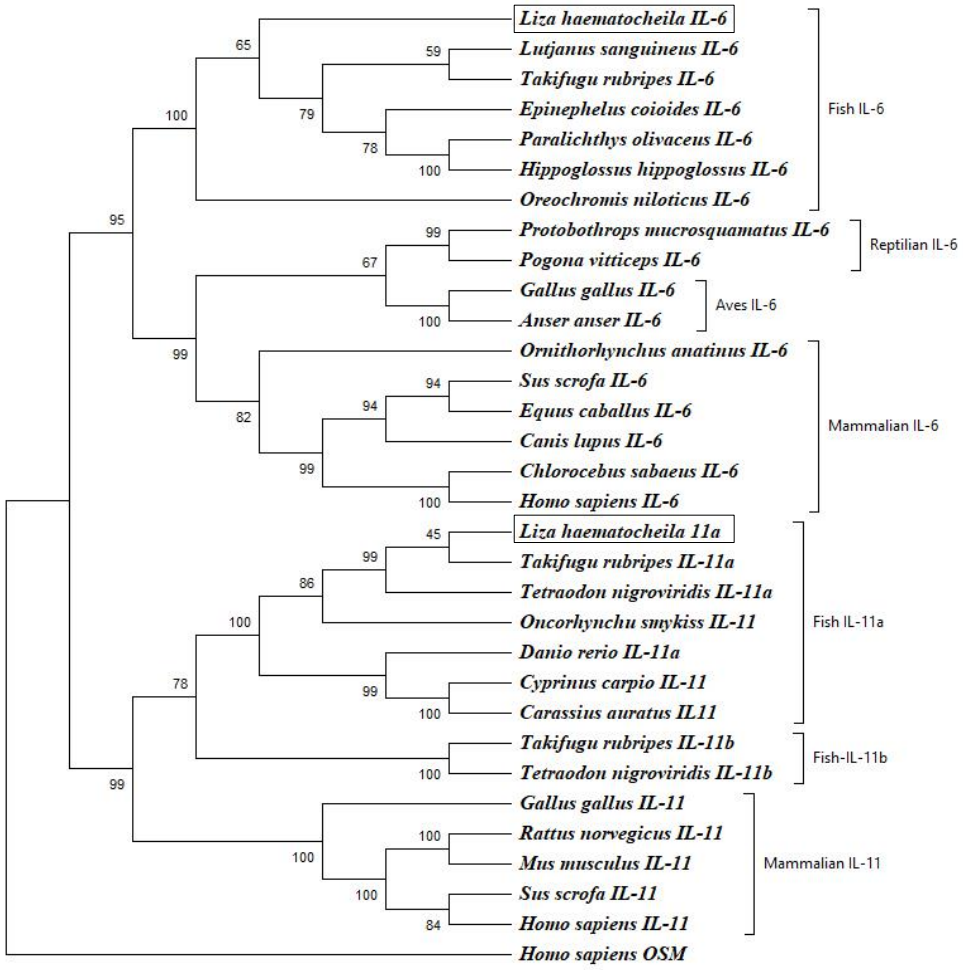


Figure 4. Phylogenetic reconstruction of

Phylogenetic reconstruction of *Lh*IL-6, *Lh*IL-11a and IL-11 sequences of different fish and species from other phyla. Bootstrap supporting values are denoted at the beginning of tree branches. The NCBI GenBank accession numbers of the amino acid sequence used in phylogenetic reconstruction are *Fundulus heteroclitus* IL-11 (XP_021178532.1), *Tetraodon*

nigroviridis IL-11a (CAI61344.1), *Takifugu rubripes* IL-11a (CAI61342.1), *Oncorhynchus mykiss* IL-11(CAI29480.1), *Cyprinus carpio* IL-11 (CAG14936.1), *Carassius auratus* IL-11 (AHJ60479.1), *Danio rerio* IL-11a (CAI61346.1), *Hippoglossus hippoglossus* IL-11b (ADP55205.1), *Paralichthys olivaceus* IL-11 (CAJ57445.1), *Takifugu rubripes* IL-11b (CAI61343.1), *Tetraodon nigroviridis* IL-11b (CAI61345.1), *Danio rerio* IL-11b (CAI61347.1), *Nanorana parkeri* IL-11 (XP_018409901.1), *Xenopus laevis* IL-11 (XP_018083580.1) , *Alligator mississippiensis* IL-11 (XP_019356430.1), *Charysemys picta* IL-11(XP_005312109.1), *Python bivittatus* IL-11 (XP_007426912.1), *Anolis carolinensis* IL-11 (XP_016851558.1), *Gallus gallus* IL-11 (XP_024998644.1), *Coturnix japonica* IL-11(XP_015706049.1), *Rattus norvegicus* IL-11 (AAK29623.1), *Mus musculus* IL-11(EDL31257.1), *Castor canadensis* IL-11 (XP_020035323.1), *Homo sapiens* IL-11 (EAW72371.1), *Oncorhynchu smykiss* IL-6 (CCV01624.1), *Danio rerio* IL-116 (NP_001248378.1), *Cyprinus carpio* IL-6 (AGR82313.1), *Oreochromis niloticus* IL-6 (XP_019205932.1), and *Epinephelus coioides* IL-6 (AFE62919.1).

3.5 Three dimensional (3D) modeling of *Lh*IL-6 and *Lh*IL-11a tertiary structure.

The predicted tertiary structures of *Lh*IL-6 and *Lh*IL-11a similarly contains a typical four-α-helix-bundle structure containing 60% of the residues of the mature peptide (Figure. 5). These topological characteristics are conserved amongst the members of class I interleukins and are important for interaction with corresponding receptors on the cell surface (Brocker et al., 2010; Czupryn et al., 1995). Several nonpolar hydrophobic side chains of leucine residues were found at the interface of α-helices, which could provide the structural stability to the mature peptide.

(A) (B)

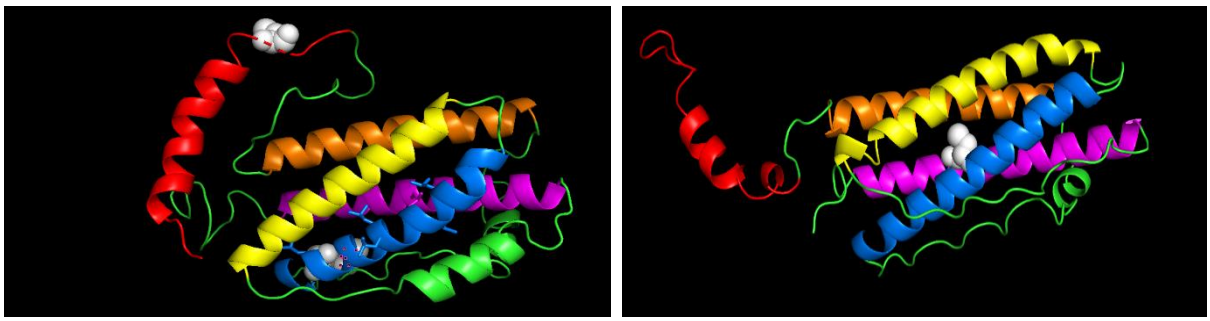


Figure 5. 3D modeling of *Lh*IL-6 and *Lh*IL-11a tertiary structure.
3D modeling of *Lh*IL-6 (A), and *Lh*IL-11a (B) tertiary structure. The tertiary structure of both peptides lateral projection, four α helices are colored, α helices A (Orange), α helices B (Yellow), α helices C (Blue), α helices D (Purple) in the main chain of the amino acid sequence

(Green). The predicted signal peptide sequence is shown in red. The cysteine residue is marked as white spheres.

3.6 Expression of *Lh*IL-6 and *Lh*IL-11a in different redlip mullet tissues

To understand the function of both interleukins in redlip mullet, the spatial expression pattern was examined in different tissues. The highest *Lh*IL-6 and *Lh*IL-11a expression were observed in the stomach and intestine (Figure 6). Similarly, in a study of rainbow trout, the intestine also had the most abundant IL-11 expression (Tafalla et al., 2016). The gastrointestinal (GI) tract of fish is a primary site of pathogenic insults encounter which may result in a proinflammatory state due to the production of inflammatory cytokines by activated macrophages such as IL-6 (Tafalla et al., 2016). As an anti-inflammatory cytokine, high constitutive expression of IL-11 could balance the inflammation and protect the GI tract from further damage in pathogenic disease. Several studies have suggested that IL-11 plays a significant role in the defense of GI diseases and repairing of the intestinal mucosa (Bozza et al., 2001; Liu et al., 1996; Orazi et al., 1996). In addition to the GI tract, high expression levels of both IL-6 and IL-11a were also found in the brain. IL-6 is considered as the main cytokine in the central nervous system (Nam et al., 2007). A similar result was reported in the study of carp IL-11 (Huising et al., 2005). The production of IL-11 in the brain might be partly due to the activated astrocytes and oligodendrocytes (Zhang et al., 2015). Murine IL-11 has been suggested to have a significant ability to limit demyelination and accelerate remyelination in the CNS (Gurfein et al., 2009; Maheshwari et al., 2013) Thus, *Lh*IL-11a might also play an important role in mullet brain.

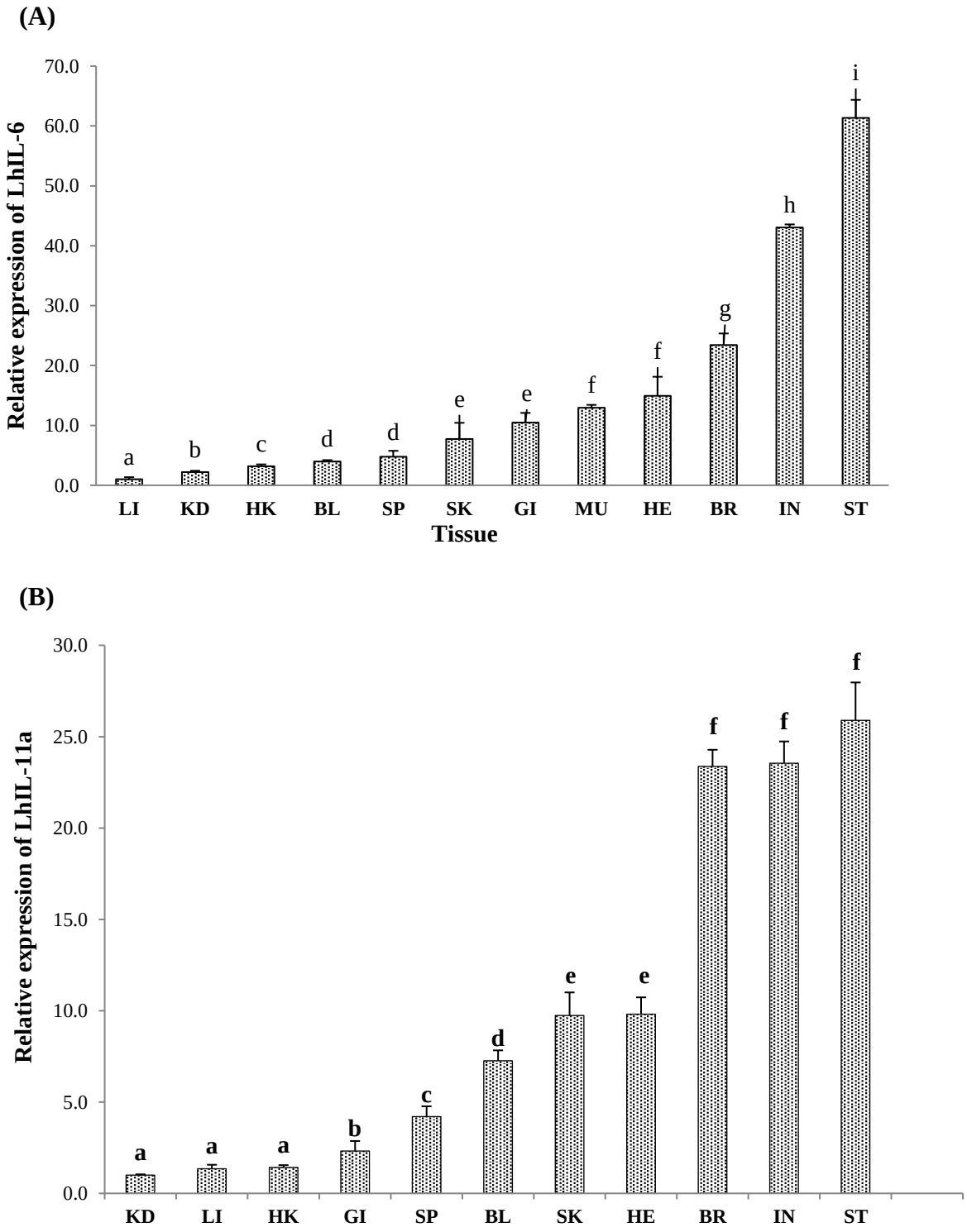


Figure 6. Tissue-specific expression of LhIL-6 (A) and LhIL-11a (B) under normal physiological condition.

The relative expression was calculated using the Livak $2^{-\Delta\Delta Ct}$ method. The mullet EF-1α gene was used as the internal reference gene. The data are represented as the mean value (n=3) ± standard deviation. The fold-changes in the tissue-specific expression were compared

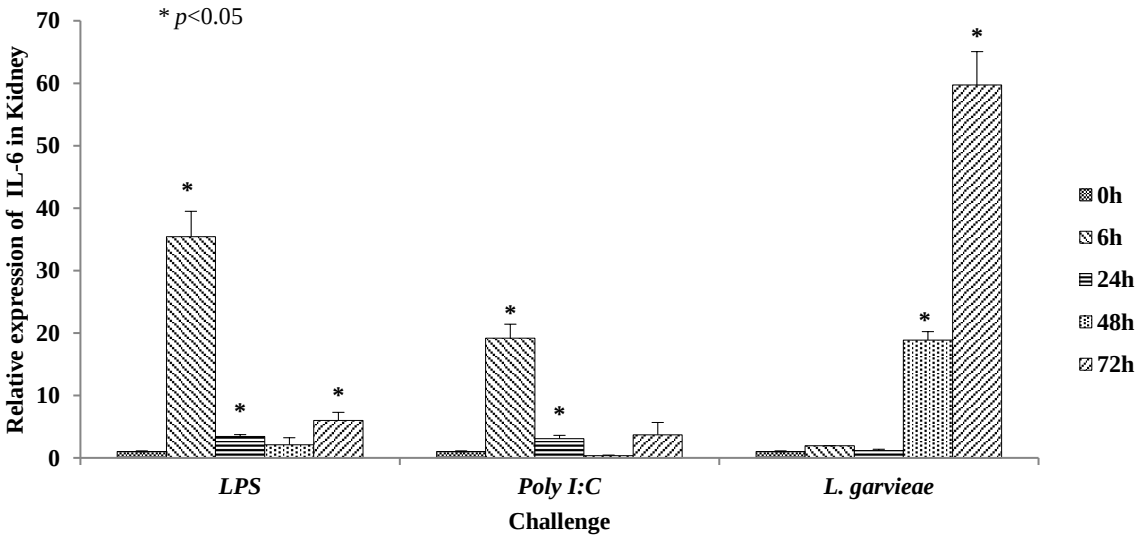
to the expression level in liver and kidney tissues respectively. A column with different letters significantly differs from each other ($p < 0.05$).

3.7 *Lh*IL-6 and *Lh*IL-11a expression under simulated pathogenic stress in mullet.

To investigate the functional role of *Lh*IL-6 and *Lh*IL-11a in the redlip mullet immune response, we examined its temporal expression profiles in PBCs and liver tissues after challenges with different immune stimulants.

LPS, poly I:C and *L. garvieae* challenges significantly course the upregulation of IL-6 in liver tissue (Figure. 7 (A)). Similarly, the expression was highly induced in LPS and Poly I:C challenge experiments in kidney tissue at early time points of the challenge. However, IL-6 expression is lower in the early time points in kidney *L. garvieae* challenge and it is highly upregulated during the later phase of the challenge.

(A)



(B)

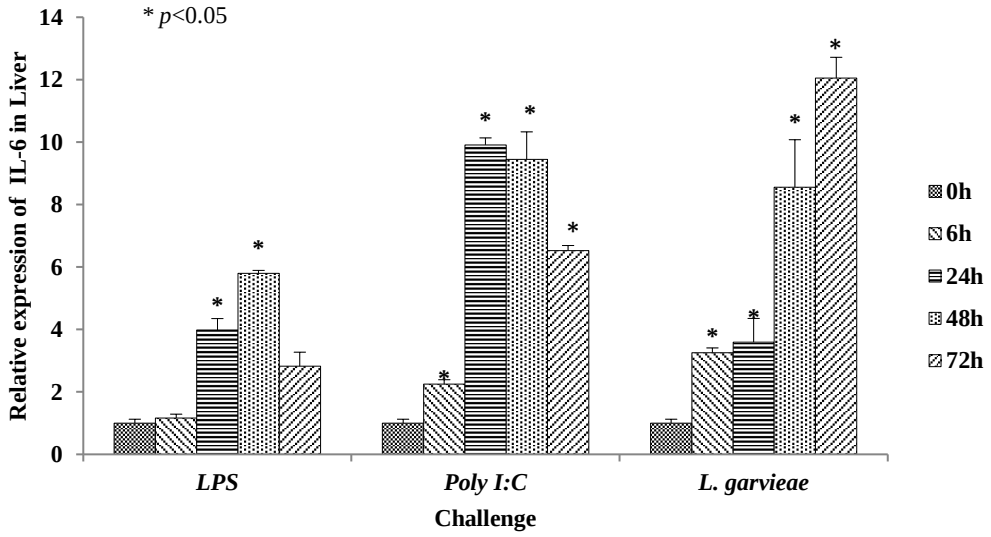


Figure 7. Temporal regulation of *Lh*IL-6 mRNA expression in the kidney (A) and liver (B) upon immune stimulation with LPS, poly I:C and *L. garvieae*.
The relative expression was calculated using the Livak $2^{-\Delta\Delta Ct}$ method. The mullet EF-1α gene was used as the internal reference gene. The data are represented as the mean value (n=3) ± standard deviation. The fold-changes in the temporal expression analysis were further normalized to the corresponding PBS-injected controls at each time point while the expression at 0-hour post-injection was used as the baseline. The column marked by an asterisk significantly differs from the expression level at 0-hour post-injection ($p < 0.05$).

LPS and poly I:C challenges significantly induced the expression level of IL-11 interleukins in both tissues, in a similar pattern of a time-dependent increase followed by a time-dependent decrease (Figure 8 (A) and (B)).

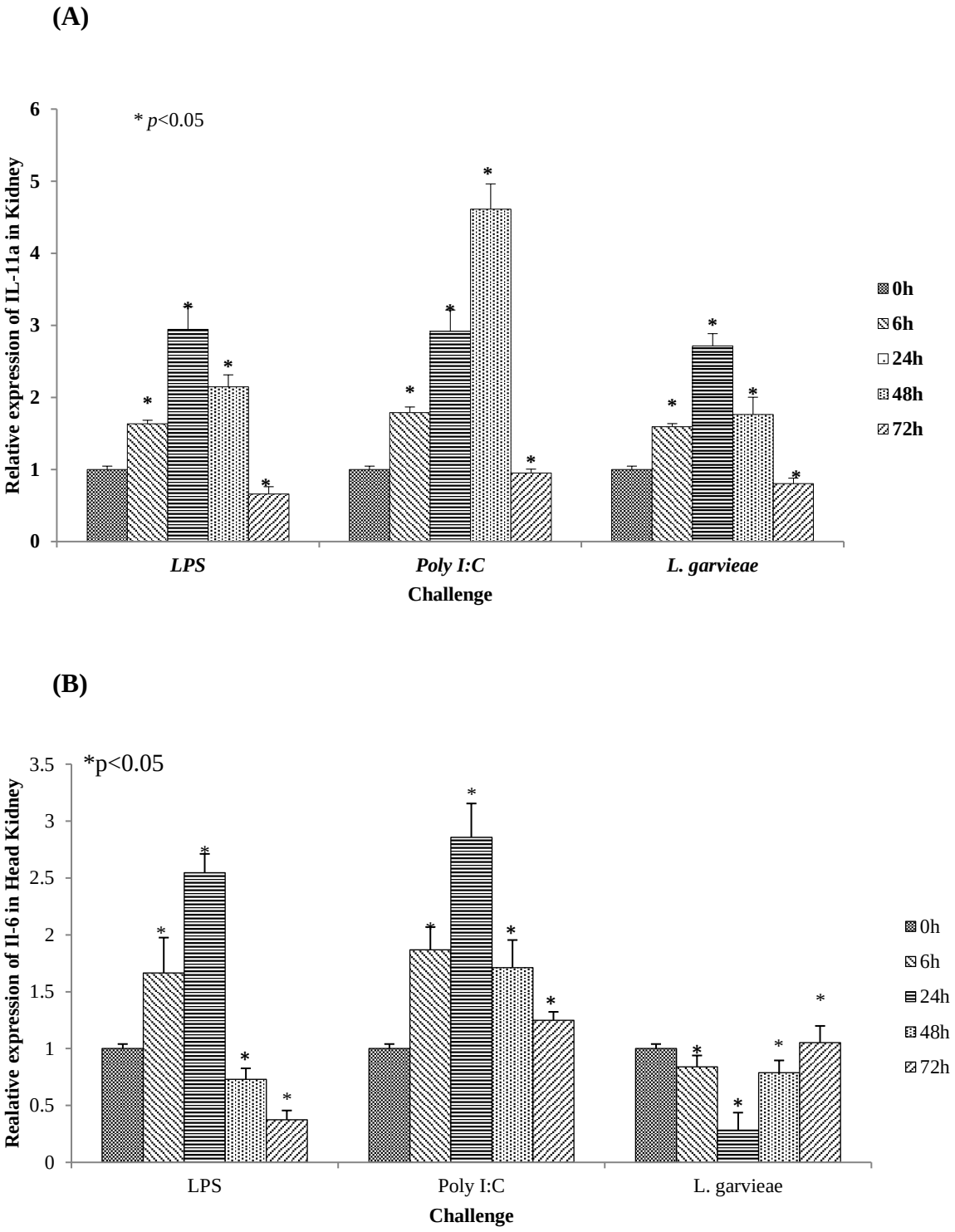


Figure 8. Temporal regulation of *Lh*IL-11a mRNA expression in liver (A) and kidney (B) upon immune stimulation with LPS, poly I:C and *L. garvieae*.

The relative expression was calculated using the Livak $2^{-\Delta\Delta Ct}$ method. The mullet EF-1α gene was used as the internal reference gene. The data are represented as the mean value (n=3) ± standard deviation. The fold-changes in the temporal expression analysis were further

normalized to the corresponding PBS-injected controls at each time point while the expression at 0-hour post-injection was used as the baseline. The column marked by an asterisk significantly differs from the expression level at 0-hour post-injection ($p < 0.05$).

LPS is a vital component of the cell wall of Gram-negative bacteria, which can stimulate the release of inflammatory cytokines and co-stimulatory molecules, including IL-6, TNFα, IL-1β, prostaglandins, interferons and macrophage-derived growth factors (McAleer and Vella, 2008). Similar to LPS, poly I:C can also induce the synthesis of inflammatory cytokines such as IL-6 (Lyons et al., 2015). The current experiment also elucidates the existing knowledge on IL-6 expression during the pathogenic invasion. Elevation of *Lh*IL-11a expression may be an attempt to ameliorate the acute phase inflammation stimulated by LPS and poly I:C. The importance of IL-11 and other IL-6 family cytokines in the protection of blood and liver cells against acute and chronic diseases has been well demonstrated in human and mouse models (Streetz et al., 2003; Tacke et al., 2009)

L. garvieae is a Gram-positive bacterial pathogen causing fatal hemorrhagic septicemia and streptococosis in many fish species (Eraclio et al., 2017; Kang et al., 2004; Morita et al., 2011). The live *L. garvieae* has affected significantly to elevate the *Lh*IL-6 expression in the later phase of both liver and kidney tissues. The initial bacterial population is smaller at the beginning and it increases the population with time. In order to control the bacterial population growth, the fish system needs to induce the inflammatory condition. Therefore, the *Lh*IL-6 transcription may be upregulated during the latter phase of the challenge.

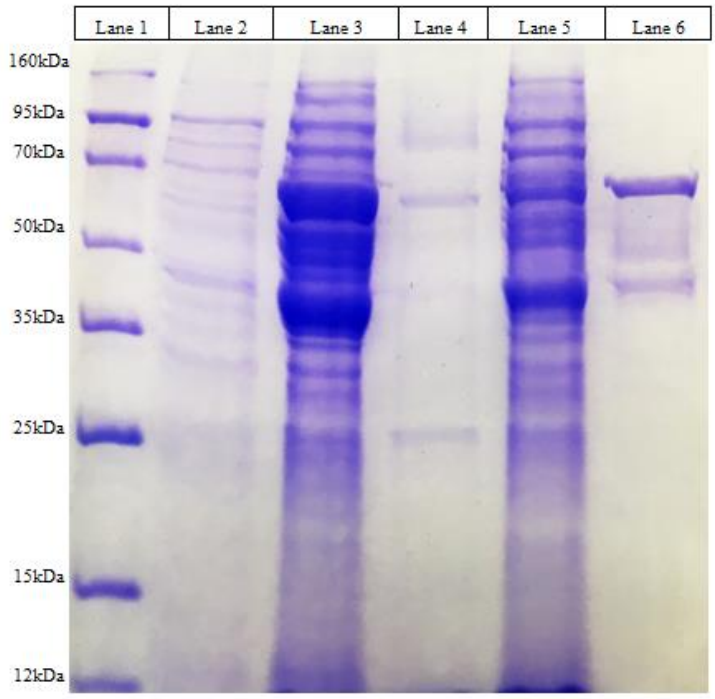
Similar to the LPS and poly I:C challenge results, after being challenged with live *L. garvieae*, *Lh*IL-11a expression was significantly upregulated in redlip mullet kidney until 48-hour post-injection (hpi). However, in the liver, the *Lh*IL-11a transcription was significantly suppressed through 48 hpi. The liver is the likely target tissue for L. garvieae infection, which causes consistent peliosis hepatis in rainbow trout and green liver syndrome in redlip mullet (Eldar and Ghittino, 1999; Han et al., 2015). The downregulation of *Lh*IL-11a expression in

the early phase of *L. garvieae* infection could contribute to maintaining an inflammatory state and the promotion of pathogen clearance in liver.

3.8 Bacterial overexpression and recombinant protein purification

Recombinant IL-6 (*rLh*IL-6) prot**e**in and IL-11a (*rLh*IL-11a) proteins were over-expressed using the pMal-c5X/ ILs construct in *E. coli* BL21 after induction with IPTG. After purifying the recombinant protein using affinity chromatography based on the presence of the maltose binding protein (MBP) fusion tag, their approximate molecular masses were determined by electrophoresis on a 12% SDS-PAGE gel using molecular mass markers (Fig. 8). The resultant *rLh*IL-6/MBP fusion protein exhibited a band of ~66 kDa being compatible with the predicted molecular mass of *Lh*IL-6 26.6 kDa; MBP 42.5 kDa (Figure. 9(A)). Moreover, the *rLh*IL-11a /MBP fusion protein had a size of ~65 kDa, including the 42.5 kDa MBP tag, confirming *rLh*IL-11a predicted the molecular mass of 25.6 kDa (Figure.9 (B)).

(A)



(B)

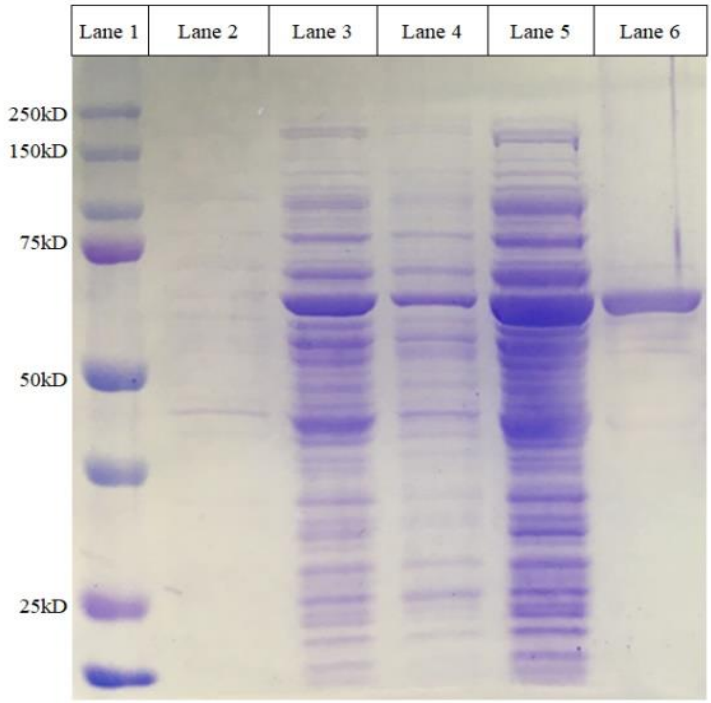


Figure 9. The SDS-PAGE analysis of purified recombinant *Lh*IL-6/MBP (A) and *Lh*IL-11a/MBP (B) fusion protein.

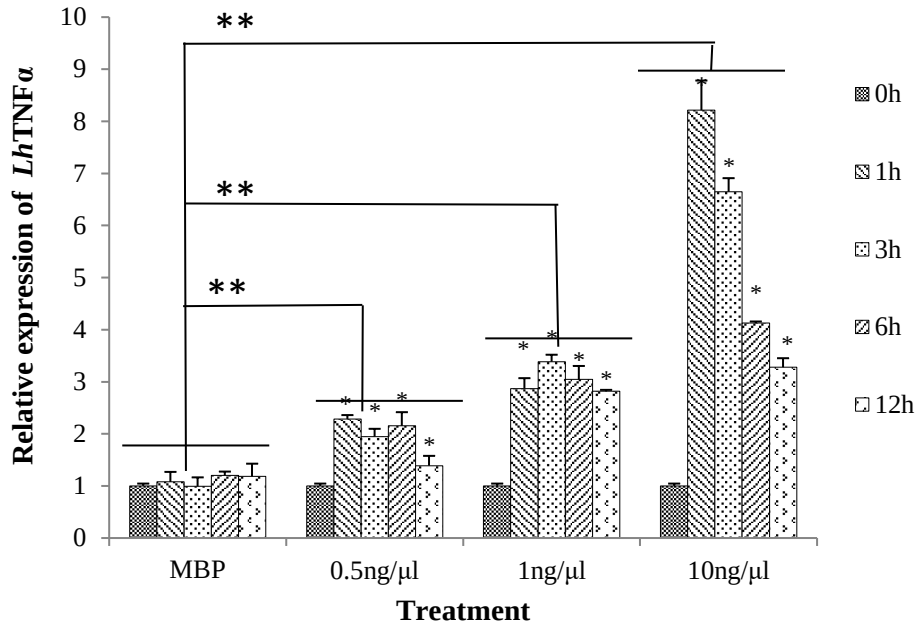
Lane 1, protein size marker; Lane 2, total soluble cellular extract from *E. coli* BL21 containing the IL/MBP fusion vector construct prior to IPTG induction; Lane 3, crude extract of IL/MBP after IPTG induction; Lane 4, pellet after centrifugation of crude lysate (IPTG induced); Lane 5, supernatant after centrifugation of crude lysate (IPTG induced); Lane 6, purified r*Lh*IL-6 (A) or purified r*Lh*IL-11a. (B)

3.9 Biological activity of rLhIL-6 and rLhIL-11a on red lip mullet kidney cells

3.9.1 Regulation of pro-inflammatory cytokine gene expression

Expression of *Lh*TNFα (Figure.10 (A)) and *Lh*IL-1β (Figure.10 (B)) in *rLh*IL-6 treated cells were significantly upregulated ($p < 0.05$) in comparison with MBP control, and shows concentration-dependent upregulation. Furthermore, the expression is reduced with time. TNFα and IL-1β are the pro-inflammatory cytokines which can intensify the inflammatory conditions in cells. The combined effect of IL-6 and other cytokines such as TNFα and IL-1β was significantly higher than when they are alone (Yokota et al., 2014). Therefore, *rLh*IL-6 may have a positive regulatory effect on other pro-inflammatory cytokines.

(A)



(B)

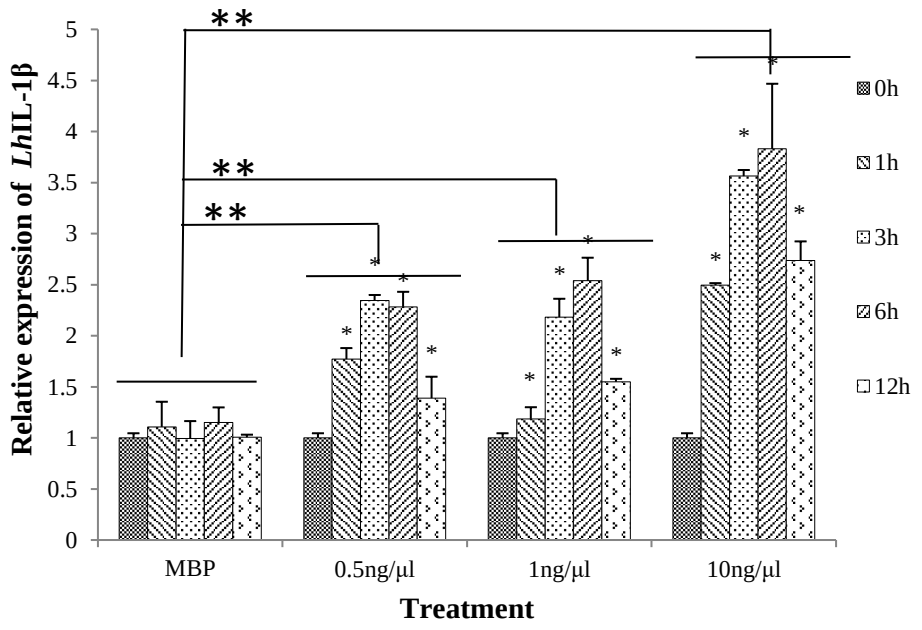
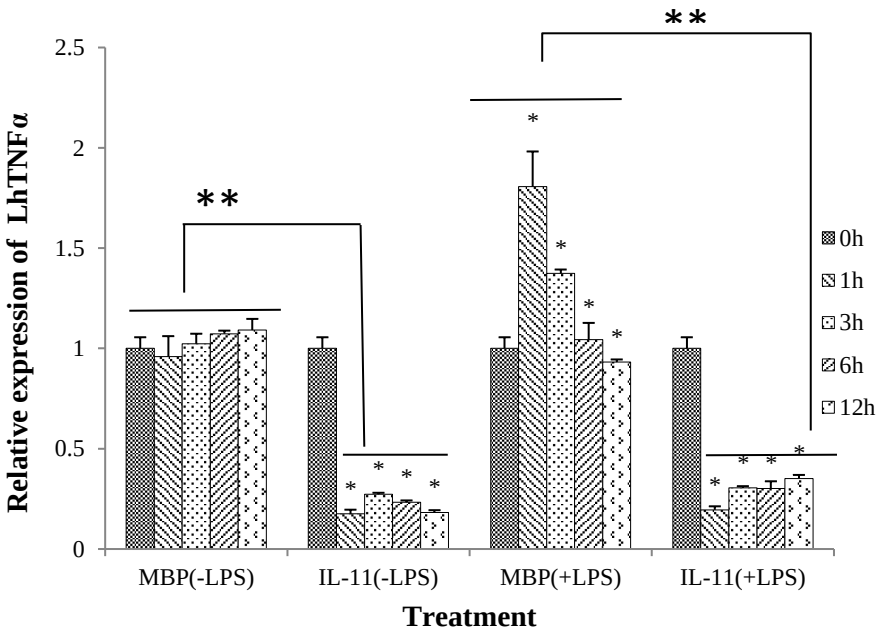


Figure 10. Effect of *rLh*IL-6 on inflammatory gene expression in mullet kidney cells.
The cells were treated with *Lh*IL-6 and MBP (with and without LPS challenge). The relative mRNA expression levels of *Lh*TNFα (A), *Lh*IL-1β (B), were examined by qRT-PCR. The asterisk (*) indicates a significant difference from expression measured at 0-hour ($p < 0.05$). Groups denoted by a double asterisk (**) significantly differ from each other ($p < 0.05$).

*Lh*TNFα expression was significantly ($p < 0.05$) up-regulated in cells treated with LPS along with MBP tag protein during the first hour of exposure and then gradually decreased close to the control level (Figure.11 (A)). In contrast, significant ($p < 0.05$) down-regulation was observed at each time point for the two treatments containing *rLh*IL-11a, regardless of whether LPS was added. Similarly, the expression of *Lh*IL-1β was also significantly ($p < 0.05$) elevated by LPS along with MBP whereas this induction was either significantly reduced or reversed to a reduction in the presence of *rLh*IL-11a (Figure. 11(B)). Additionally, a time-dependent down-regulation of *Lh*IL-1β expression was observed in the treatment with *rLh*IL-11a alone.

(A)



(B)

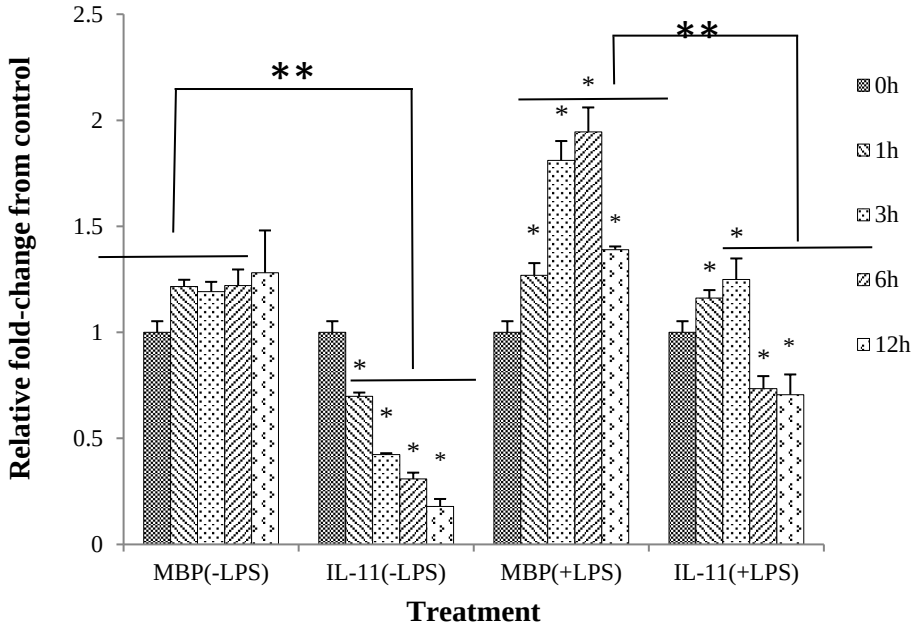


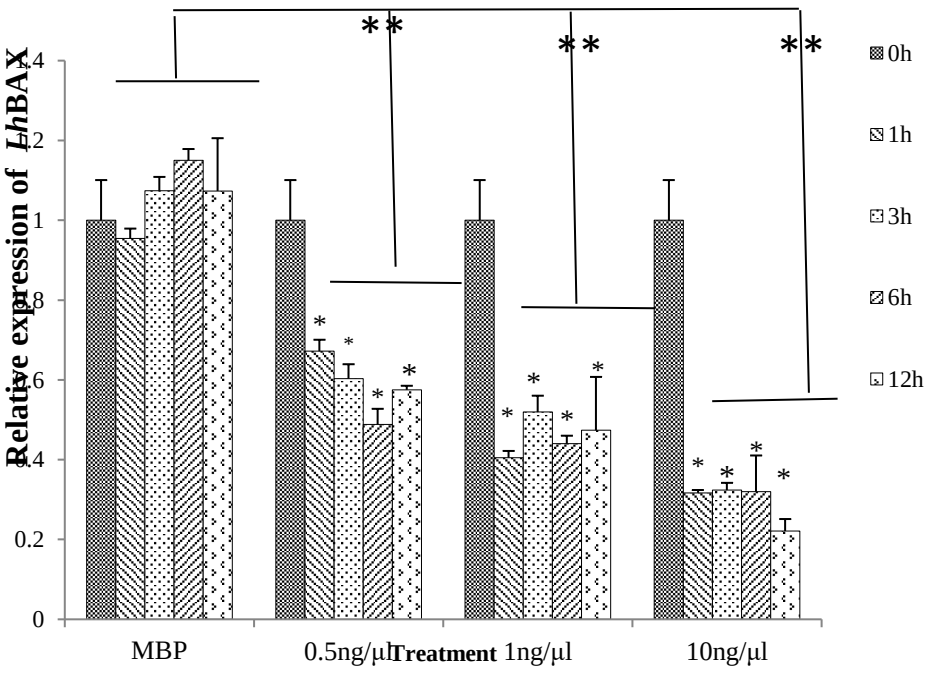
Figure 11. Effect of *rLh*IL-11 on inflammatory gene expression in mullet kidney cells.
The cells were treated with *Lh*IL-11a and MBP (with and without LPS challenge). The relative mRNA expression levels of *Lh*TNFα (A), *Lh*IL-1β (B), were examined by qRT-PCR. The asterisk (*) indicates a significant difference from expression measured at 0-hour ($p < 0.05$). Groups denoted by a double asterisk (**) significantly differ from each other ($p < 0.05$).

Our results suggest that *rLh*IL-11a can reduce LPS stimulated inflammation and suppress the constitutive expression of inflammatory cytokines in redlip mullet cells. Pro-inflammatory cytokines are inducible by LPS, as part of the innate immune response to bacterial infection (Silva et al., 2018; Vanoni et al., 2017). However, the excessive production of pro-inflammatory cytokines may damage the host tissue and lead to a systemic inflammatory response syndrome, such as septic shock. Anti-inflammatory cytokines, such as IL-11, have been demonstrated to play crucial roles in attenuating excessive inflammation and maintaining immune homeostasis (Aziz et al., 2012).

3.9.2 Regulation of anti-apoptotic BCL2 and pro-apoptotic BAX gene expression

BCL2 and BAX are two prototypical members of the BCL2-like family, which play anti-apoptotic and pro-apoptotic roles, respectively (Cui and Placzek, 2018; Lee et al., 2014). Significant downregulation of *Lh*BAX transcription ($p < 0.05$) was observed in *rLh*IL-6 treated mullet kidney cells. Moreover, the expression shows concentration-dependent downregulation of *Lh*BAX (Figure. 12(A)). The expression of *Lh*BCL2 in rLhIL-6 treated cells were significantly upregulated ($p < 0.05$) in a concentration-dependent manner compared with the expression of MBP treated cells. In each experiment group, an increase of transcription at the early phase and decrease in late phase is observed. BAX is the pro-apoptotic member of the BCL2 family. Elevated expression of the gene results in the apoptosis of target cells. In order to prevent the cells from apoptosis, the BAX gene expression would be in fine tune with other anti-apoptotic proteins (Antonsson et al., 1997). Here in the current experiment, the downregulation of LhBAX may be the result of self-attempt to reduce the apoptotic effect by the cells due to the high concentration of inflammatory IL-6. In contrast, the BCL2 gene has upregulated with IL-6 treatment(Figure. 12(B)). There may be due to an attempt to ameliorate the cell condition due to the inflammation caused by the IL-6.

(A)



(B)

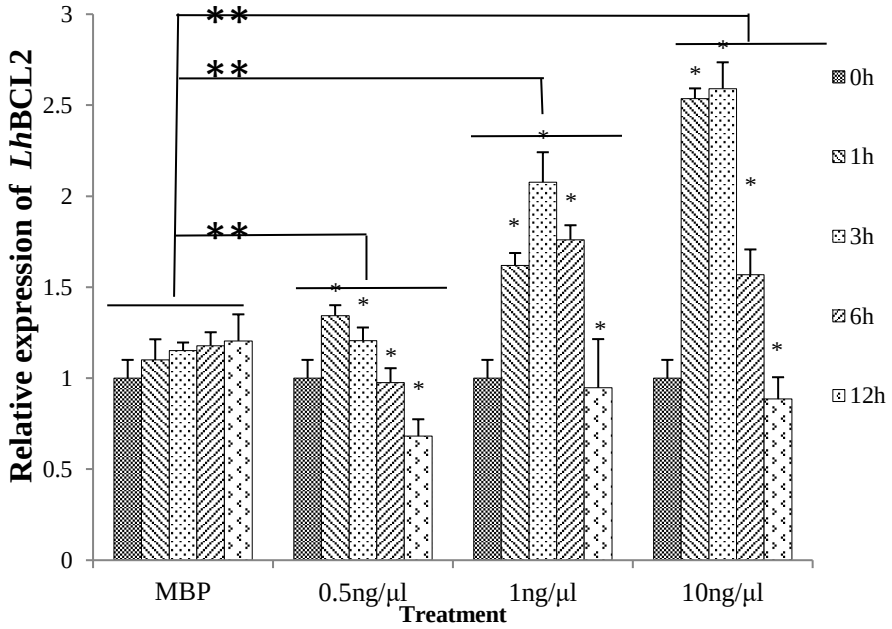
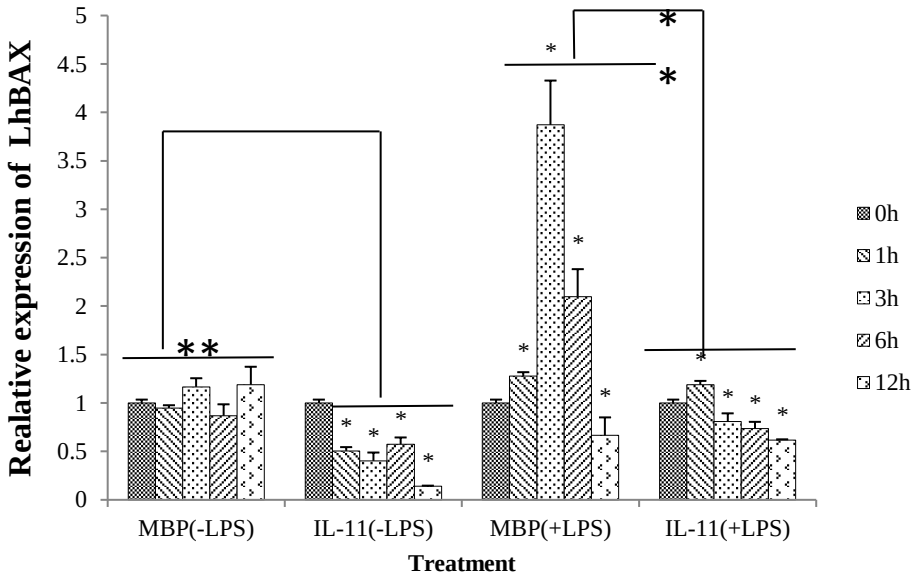


Figure 12. Effect of *rLh*IL-6 on apoptotic gene expression in mullet kidney cells.
The cells were treated with *Lh*IL-11a and MBP (with and without LPS challenge). The relative mRNA expression levels of *Lh*BAX (A), *Lh*BCL2(B), were examined by qRT-PCR. The asterisk (*) indicates a significant difference from expression measured at 0-hour ($p < 0.05$). Groups denoted by a double asterisk (**) significantly differ from each other ($p < 0.05$).

Interestingly, despite opposite functions in apoptosis, these two genes in redlip mullet showed a similar transcriptional response to LPS and *rLh*IL-11a treatments (Figure.13). The expression of both genes was significantly ($p < 0.05$) induced in the treatment of LPS along with MBP, while in the treatment of LPS along with *rLh*IL-11a the induction effect of LPS was significantly ($p < 0.05$) blunted. Moreover, significant ($p < 0.05$) down-regulation of *Lh*BCL2 and *Lh*BAX expression was observed at almost all of the time points in the treatment with only *rLh*IL-11a.

(A)



(B)

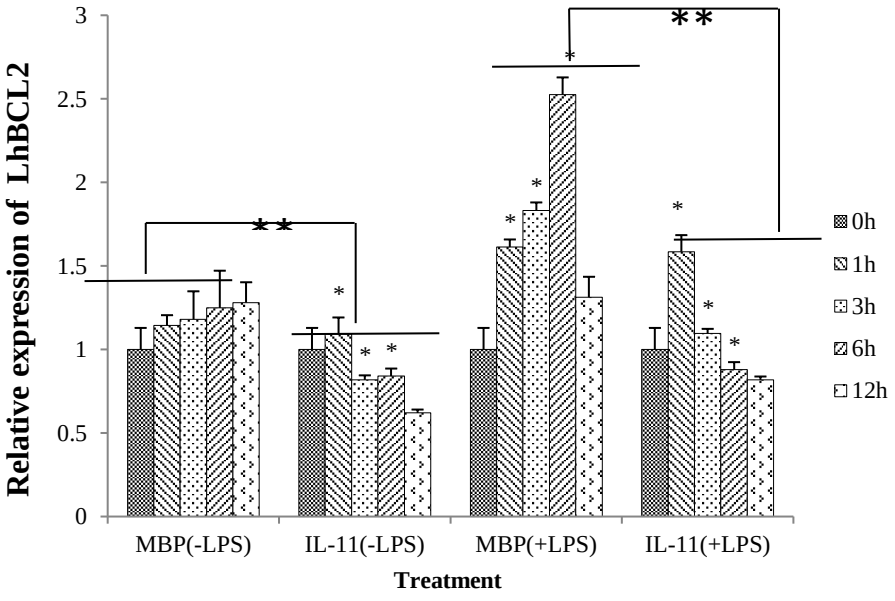


Figure 13. Effect of *rLh*IL-11a on apoptotic gene expression in mullet kidney cells.

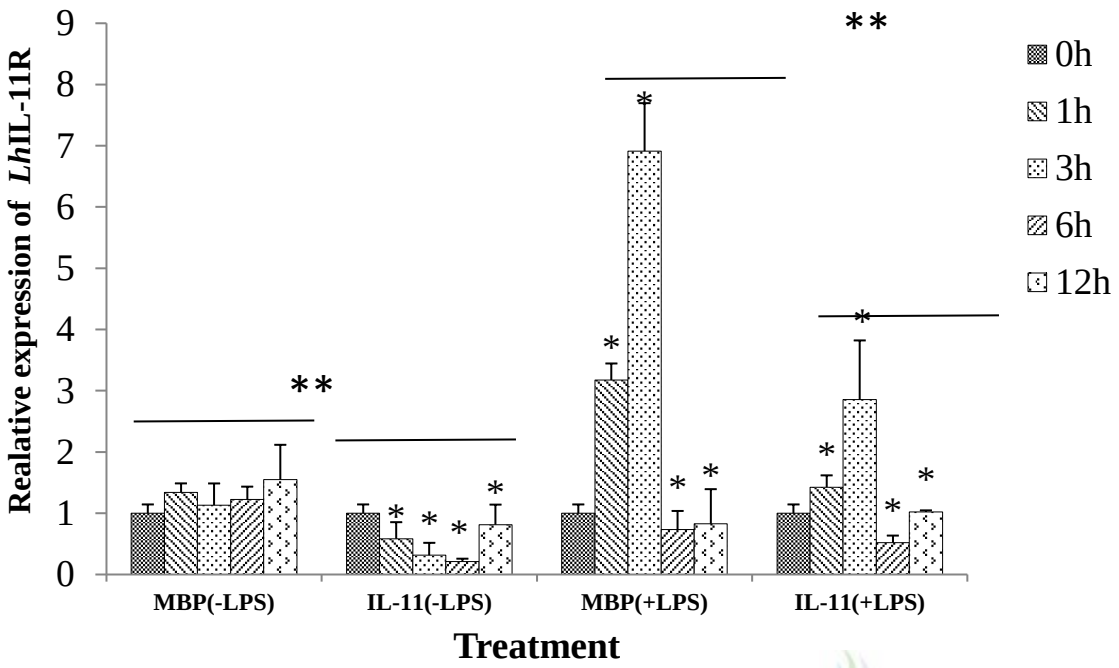
The cells were treated with *Lh*IL-11a and MBP (with and without LPS challenge). The relative mRNA expression levels of *Lh*BAX (A), *Lh*BCL2(B), were examined by qRT-PCR. The asterisk (*) indicates a significant difference from expression measured at 0-hour ($p < 0.05$). Groups denoted by a double asterisk (**) significantly differ from each other ($p < 0.05$).

The apoptotic fate of cells is determined by the BCL2/BAX ratio (Salakou et al., 2007). This ratio was significantly decreased at the 3-hour post the treatment of LPS along with MBP, indicating an apoptosis-induction effect of LPS. Nevertheless, in the two treatments containing *rLh*IL-11a, the BCL2/BAX ratios were significantly elevated at most of the time points, indicating that the apoptosis was suppressed by *rLh*IL-11a (Fukamachi et al., 1998). Similarly, in a study of the mouse, IL-11 was also reported to protect fibroblast cell against FAS-induced apoptosis through increasing BCL2/BAX ratio (Moodley et al., 2003). These results suggest *Lh*IL-11a may have an anti-apoptosis function in redlip mullet.

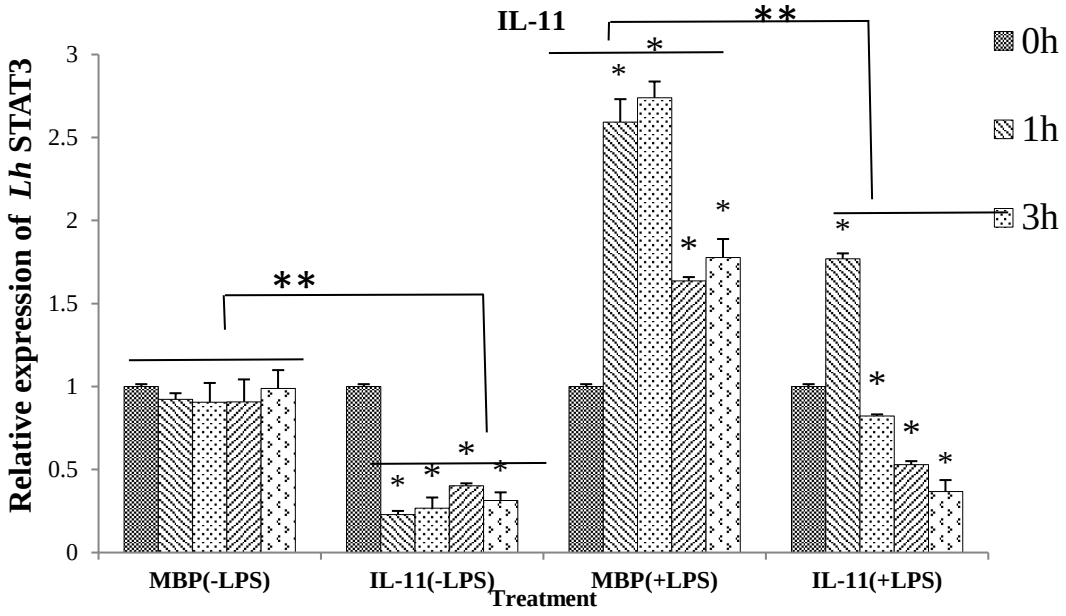
3.9.3 Feedback transcription response of STAT3 signaling cascade components to IL-11a ligand

Transcription of all the three principal components of IL-11/STAT3 signaling cascade namely, the receptor of *Lh*IL-11 (IL-11R), *Lh*STAT3, and *Lh*SOCS3 gens has significantly downregulated ($p < 0.05$) in the *rLh*IL-11a treated cells (Figure.14). *Lh* IL-11R transcription has upregulated by the LPS. The results indicate that LPS treatment can stimulate IL-signaling. Interestingly, transcription of the suppressor of cytokine signaling 3 (SOCS3) gene also upregulated by the LPS treatment in similar to the other components.

(A



(B)



(C)

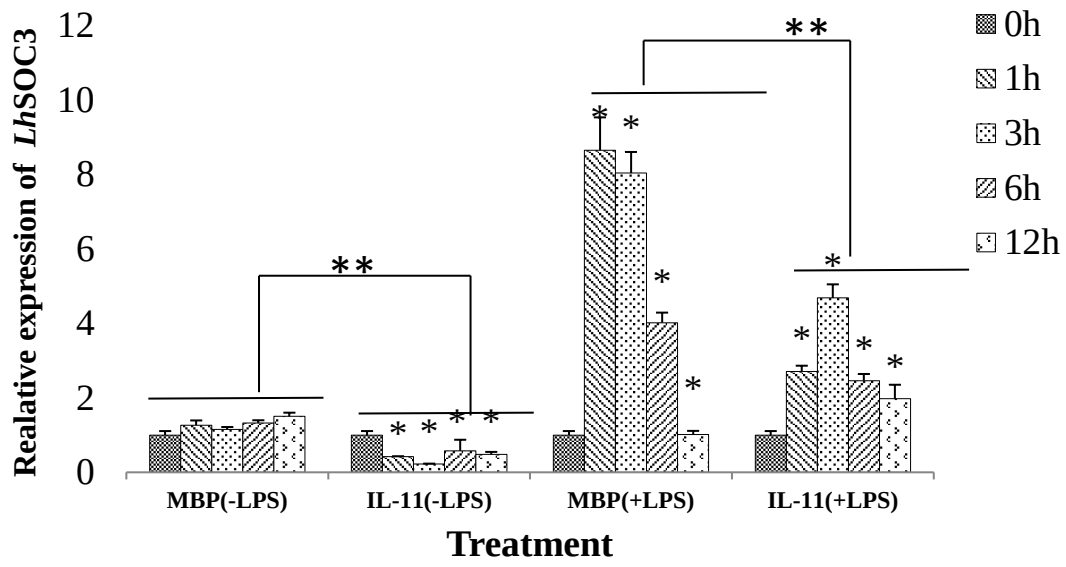


Figure 14. Effect of *rLh*IL-11a on the expression of STAT3 signaling cascade components in mullet kidney cells.

The cells were treated with *Lh*IL-11a and MBP (with and without LPS challenge). The relative mRNA expression levels of *Lh*IL-11R (A), *Lh*STAT3 (B) and *Lh*SOCS3 (C) were examined by qRT-PCR. The asterisk (*) indicates a significant difference from expression measured at 0-hour ($p < 0.05$). Groups denoted by a double asterisk (**) significantly differ from each other ($p < 0.05$).

To prevent overstimulation of target genes, nature has invented sophisticated mechanisms to turn off cytokine-mediated signal transduction. SOCS 3 transcription is upregulated with activation of STAT3, in pro-inflammatory IL-6 signaling (Heinrich et al., 2003). On the other hand, anti-inflammatory IL-11 may have a different mechanism to downregulate SOCS 3, in order to facilitate the upregulation of target anti-inflammatory genes. In contrast, The STAT 3 signaling may have been controlled by downregulation of IL-11R receptor and STAT 3. The IL-11R receptor is encircled by the plasma membrane and subjected to lysozyme mediated degradation (Lokau et al., 2017). Phosphorylated STAT3 also subjected to proteasome degradation in the nucleus after the DNA binding (Heinrich et al., 2003). These mechanisms enable the further controlling of induction of IL-11 signaling. Similarly, downregulation of IL-11R and STAT3may be used to control the further induction of IL-11 signaling in mullet kidney cells in the current experiment.

3.10 NO production assay in murine macrophage (RAW264.7) cells

The NO production in LPS treated cells was dramatically increased in the presence of MBP or heat-denatured r*Lh*IL-6 protein, compared to that in the untreated cells. In the presence of *rLh*IL-6 ligand, NO production was significantly ($p < 0.05$) increased in the r*Lh*IL-6 treatment in a dose-dependent manner (Figure. 15(A)). In a similar experiment, the NO production in LPS treated cells was dramatically increased in the presence of MBP or heat-denatured r*Lh*IL-11a protein, compared to that in the untreated cells. However, in the presence of *rLh*IL-11a, the LPS stimulated NO production was significantly ($p < 0.05$) reduced in a dose-dependent manner (Figure. 15 (B)).

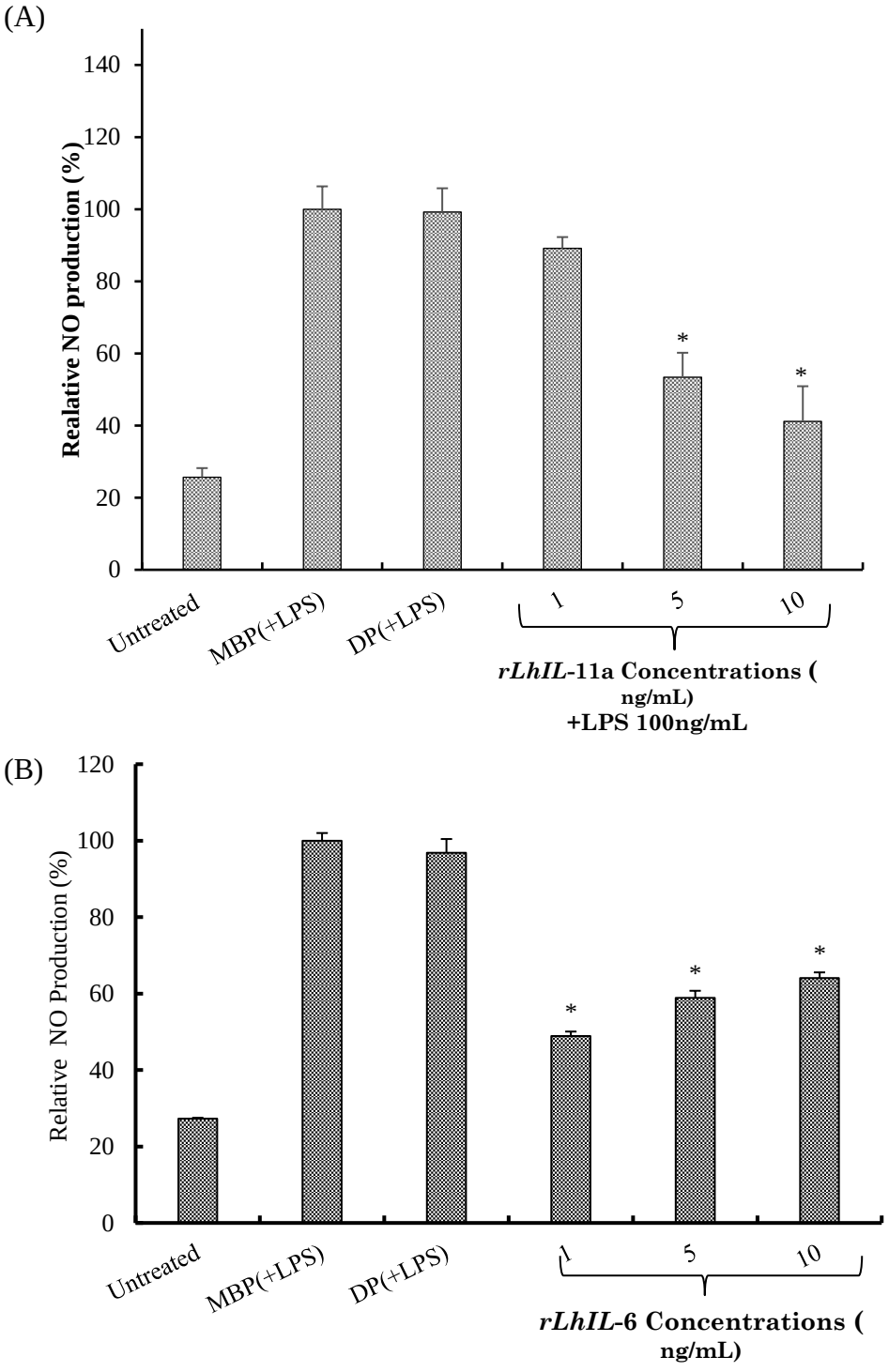


Figure 15. Effect of rLhIL-6 and rLhIL-11a on Nitric oxide (NO) production in RAW264.7 murine macrophage cells.

Effect of r*Lh*IL-6 on Nitric oxide (NO) production in RAW264.7 murine macrophage cells (A). Effect of r*Lh*IL-11a on Nitric oxide (NO) production in RAW264.7 murine macrophage cells (B). Absorbance was measured at 540 nm and the assay was conducted in triplicate. The significant differences from MBP treated cells are indicated with an asterisk (*, $p < 0.05$).

NO is an important mediator and regulator of the inflammatory response (Tripathi et al., 2007). The biosynthesis of NO in cells is regulated mainly by the inducible nitric oxide synthase (iNOS) gene, and iNOS expression can be highly stimulated upon activation of NF-κB transcription factors in response to many stimuli, including pro-inflammatory cytokines such as IL-6, LPS, poly I:C and other pathogenic components (Morris et al., 2003). In the current experiment, the recombinant IL-6 shows the ability to induce the iNOS gene and produce nitric oxide. On the other hand, a high level of NO can cause irreversible inhibition of the respiratory chain and eventually lead to severe oxidative stress and cell death (Brown, 2001). In previous studies, several anti-inflammatory cytokines, such as IL-4, IL-10, and IL-11, have shown the ability to attenuate iNOS activation and to prevent the overproduction of NO (Cattaruzza et al., 2003; Patton et al., 2002; Trepicchio et al., 1996). Our results suggest that *Lh*IL-11a might have an inhibitory function in NO synthesis regulation as other anti-inflammatory cytokines.

4. Conclusion

The current experiment is the first attempt to clone and functionally characterize teleostean IL-6 IL-11a genes. Both *Lh*IL-6 and *Lh*IL-11a has conserved structural features and close phylogeny with other IL-6 and IL-11a orthologs respectively. Transcription of *Lh*IL-6 and *Lh*IL-11a mRNA were significantly activated in response to bacterial and viral stimuli, indicating an important role in mullet host defense against pathogenic infection. Moreover, both r*Lh*IL-6 and r*Lh*IL-11a protein demonstrated a significant regulatory effect on the genes involved in inflammation and apoptosis in redlip mullet cells. Effect of over expression of r*Lh*IL-11a on STAT3 signalling cascade componants were analysed and the results revalved that r*Lh*IL-11a has critical of feedback regulaton for the cascade componants. The recombinant *Lh*IL-6 protein stimulates the production of nitric oxide and *Lh*IL-11a protein also displayed a strong inhibitory effect on NO production. Further studies are needed to fully elucidate the regulatory mechanism of fish IL-11a in inflammation, apoptosis and other crucial immune responses.

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