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Toll-like receptor (TLR) 4 in mrigal (*Cirrhinus mrigala*) : Response to lipopolysaccharide treatment and *Aeromonas hydrophila* infection

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Abstract

Toll-like receptors (TLRs) are one of the key components of innate immunity. Among various TLRs, TLR4 plays the central role in recognition of bacterial lipopolysaccharides (LPS), a component of the outer membrane of Gram-negative bacteria. In this article, we identified TLR4 gene in the Indian major carp (IMC) mrigal (Cirrhinus mrigala), a highly commercially important fish species in the Indian subcontinent. Ontogeny analysis of TLR4 gene by quantitative real-time PCR (qRT-PCR) assay revealed its constitutive expression in all embryonic developmental stages. The tissue specific expression analysis of TLR4 by qRT-PCR revealed wide distribution of TLR4 in various organs/tissues of mrigal fingerlings. The highest expression of TLR4 was observed in skin and the lowest expression was in muscle. Stimulation of mrigal fingerlings with LPS activated TLR4 signaling resulting in significant ($p < 0.05$) induction of the effector molecule IL-10 in the treated fish group as compared to their control. Moreover, inductive expression of TLR4 and IL-10 genes in all tested tissues of mrigal were also observed following a Gram-negative bacterial (Aeromonas hydrophila) infection. These findings together highlighted the important role of TLR4 in immune surveillance of various organs during pathogenic invasion in fish.

Keywords: *Cirrhinus mrigala*, TLR4, IL-10, LPS, *Aeromonas hydrophila*

Introduction

Innate immunity is the fundamental defense strategy against infections and is considered as the most primitive and universal form of host defense[1]. Higher vertebrates like birds and mammals show higher level of immune defense mechanism like adaptive immunity, whereas insects and lower vertebrates like fish and amphibian primarily depend upon innate immunity, a faster but less specific than adaptive immunity[2-3]. Innate immune system recognizes pathogens or conserved pathogen derived structures like lipoproteins, peptidoglycan (PGN), lipoteichoic acid (LTA), zymosan, heat shock protein (hsp), lipopolysaccharides (LPS), flagellin, CpG-DNA and nucleic acids of the micro-organisms (microbes/pathogen associated molecular patterns, MAMPs or PAMPs) by germ-line-encoded pattern recognition receptors (PRRs) that are distributed in cell surface, intracellular compartments, or secreted into the blood stream and tissue fluids[4]. Three major classes of PRRs have been identified: toll-like receptors (TLRs) that recognize ligand on either the extracellular surface or within the endosome, NOD-like receptors (NLRs) that function as cytoplasmic sensors and RIG I-like receptors (RLRs) that recognize viruses[2, 5-6].

Toll-like receptors (TLRs) are a family of PRR that are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals, and sense a diverse range of PAMPs for initiation of a well-coordinated immune response to limit or eliminate invading microbes[4, 7]. They are classified into various types, depending upon their primary sequence and types of ligands (PAMPs) recognition[2]. Stimulation of TLRs by PAMPs initiates subsequent signaling pathway resulting in the induction of innate immunity genes expressions.

LPS, a major component of the outer membrane of Gram negative bacteria, is recognized by TLR4 as ligand. In mammals, TLR4 transmits signals through several intracellular TIR domain containing adaptor molecules to initiate proinflammatory gene expressions[8-9]. Two pathways are known to be involved in TLR4 downstream signaling viz. the MyD88 dependent pathway and TIR domain containing adaptor inducing IFN-β (TRIF)-dependent pathway. In MyD88-dependent signaling pathway, ligand binding to TLR triggers sequential recruitment of MyD88, members of IRAK (interleukin-1 receptor-associated kinase) family and TRAF6 that resulted in nuclear factor (NF)-κB activation and induction of several inflammatory cytokines. The second pathway for TLR4 signaling requires TRIF (also known as TICAM1) and TRIF-related adaptor molecule (TRAM; also known as TICAM2) for transmission of the signal to activate IFN-regulatory factor 3 (IRF3) and to induce the expression of interferons and co-stimulatory molecules[10]. In fish, however, LPS recognition and sensitivity are essentially different than mammals. Most of the fishes lack TLR4 orthologs as well as the essential co-stimulatory molecules i.e., myeloid differentiation protein 2 (MD-2) and CD14 for TLR4 activation by LPS[11]. Till date piscine TLR4 gene has been identified in zebrafish, and some other cyprinidae like rare minnow and common carp[12-14].

Mrigal (*Cirrhinus mrigala*) is one of the most commercially important and highly favored freshwater-cultured fish species in

India, but the fish farming industry suffered from considerable economic losses due to the diseases caused mostly by various Gram-negative bacteria belonging to the genus *Aeromonas*, *Pseudomonas*, *Edwardsiella* and *Flavobacterium*[15-18]. As compared to higher vertebrates, the adaptive immune system in fish is less developed, and to protect themselves against diseases, they primarily depend on their innate immune response elicited by various PRRs. In mrigal, information on various PRRs is inadequate, and there is very little information on any TLR gene and their function. Therefore, the present study was undertaken to identify TLR4 gene in mrigal, to study their expression pattern in embryonic developmental stages, in various organs, and to investigate their role in LPS stimulation and during Gram-negative bacterial *(Aeromonas hydrophila)* infections.

Material and Methods

Fish: Mrigal (*Cirrhinus mrigala*) weighing ~ 50 g was obtained from the Central Institute of Freshwater Aquaculture (CIFA), and was stocked in 500 L aerated tanks, with each tank containing 50 fishes. Before the start of the experiment, acclimatization was carried out for 3-weeks, and the fish were fed twice a day with commercial carp diet with daily two-third water exchange. The water temperature varied from 25 to 28 ^{o}C and the pH of the water varied from 7.4 to 7.6 during the experiment.

Bacteria: *A. hydrophila* (ATCC-35654) was cultured in LB broth (USB, USA) at 37 ^{o}C for 16 h with constant shaking. Viable count was determined as colony forming unit (CFU) following 10 fold serial dilutions and plating on nutrient agar.

Cloning of mrigal TLR4: To clone TLR4 gene in mrigal, total RNA was extracted from gill and cDNA was prepared following the protocol as described later. For PCR amplification, degenerate primers (T4-FW and T4-RV; table-1) were designed based on the nucleotide sequences of the conserved regions of zebrafish (GenBank ID: NM_212813.1), mouse (GenBank ID: NM_021297.2) and chicken (GenBank ID: NM_001030693.1) TLR4 gene. The PCR was carried out in a 50 µl reaction volume with 1 µl of the cDNA as template under the conditions of one cycle of initial denaturation at 94°C for 2 min followed by 45 cycles of 94ºC for 30 sec, 52ºC for 30 sec, 72 ºC for 1 min and a final extension at 72ºC for 5 min. One-fifth of the PCR products was analyzed in 2% agarose gel, and the single specific band was purified with agarose gel DNA extraction kit (Roche, Germany). The eluted DNA was cloned in pGEM-T Easy vector (Promega, Madison, USA) and both strand DNA sequencing was carried out with T7 and SP6 primer following Sanger sequencing protocol in 96 capillary high through put sequencer, ABI 3730 XL (Xcelris Labs Ltd, Ahmedabad, India). The obtained DNA sequence was analyzed through BLAST search[19] for its identification and confirmation.

***In vivo* expression of mrigal TLR4 gene:** To investigate TLR4 gene expression during various developmental stages of mrigal, mature mrigal were bred by induced breeding techniques. Fertilized eggs were collected from CIFA hatchery, and were maintained at ambient temperature (28-29°C). Periodical observation was carried out under the inverted microscope to identify various developmental stages from fertilized eggs to hatchlings. Samples were collected separately at different hours of developments viz. 0 h (fertilized egg), 5, 10, 15 h, and hatchlings at 20 h. Total RNA was extracted using TRIzol reagent, cDNA was prepared using oligo-dT primer, and quantitative real-time PCR (qRT-PCR) was carried out to analyze TLR4 gene expression keeping β-actin as an internal control.

To study the basal expression of TLR4 in various tissues, we separately collected gill, liver, kidney, intestine, heart, brain, spleen, muscle, skin, eye and blood from mrigal fingerlings in TRIzol reagent. Total RNA was isolated from each sample, and cDNA was prepared. Quantitative real-time PCR was carried out to analyze TLR4 gene expression in various tissues, keeping β-actin as an internal control. To eliminate individual variations, samples from each fish were analyzed separately by qRT-PCR and their mean value was considered.

Table-1
Primers, their sequence and application

Target gene	Primer	Sequence (5'→3')	Annealing temp (°C)	Amplicon size (bp)	GenBank ID	Application
TLR4	T4-FW	TGGTGTMKCTTTGARTWTGA	52	204	-	Cloning
	T4-RV	TTTCCTGAGTCGTATCCAGAA				
TLR4	TLR4 Fw	TTAGCTCAGTCTCGCTTTATGATGG	55	146	GU248419	Real-time PCR
	TLR4 Rv	GGGTTTTTGCTCCATTTTAGGTACG				
IL-10	IL-10 Fw	GCGCAGTGCAGAAGAGTCGAC	55	310	HM228926	
	IL-10 Rv	CCCGCTTGAGATCCTGAAATATA				
β-actin	β-actin Fw	AGACCACCTTCAACTCCATCATG	55	200	EU184877	
	β-actin Rv	TCCGATCCAGACAGAGTATTTACGC				

***In vivo* ligand exposure and bacterial challenge:** Healthy mrigal fingerlings were divided into control and treated groups keeping three fish in each group. For ligands exposure, purified LPS of *Escherichia coli* (serotype O55: B5) (SIGMA, Germany) was diluted in endotoxin free water at 5 mg/ml, and 100 µl of endotoxin free water containing 20 µg of diluted LPS was injected to the fish by intra-venous (i.v.) route through caudal vein (treated group). The control fish group was i.v. injected only with 100 µl of endotoxin free water. For bacterial infection, fish were intra-peritoneally (i.p.) injected with 100 µl of PBS containing *A. hydrophila* (4×10^6 CFU/fish) and were designated as treated fish group. The control fish group was injected with 100 µl of PBS only, and kept separately in the aerated tank. After 4 h of LPS treatments and 6, 12 and 24 h of bacterial infections, control and treated group of fish were sacrificed and tissues were collected separately in TRIzol reagent. Total RNA was isolated, cDNA was prepared and was kept at -80 ^{o}C till further analysis.

RNA isolation and 1st strand cDNA synthesis: From the TRIzol treated samples, *viz.,* gill, liver, kidney, spleen, intestine, skin, heart, brain, blood, muscle and eye, total RNA was extracted following the standard protocol (Invitrogen, USA). The concentration of the total RNA was measured by UV-spectrophotometer (Eppendorf, India) and the integrity of the RNA was assessed by observing the band intensity of 28 and 18S ribosomal RNA on 1 % agarose gel. To synthesize 1st strand cDNA, 1 µg of total RNA was treated with 1 U of DNase I (MBI, Fermentas, USA) and reverse transcription was carried out using oligo-dT primer and RevertAid 1st strand cDNA synthesis kit (MBI, Fermentas, USA). The confirmation of cDNA synthesis was carried out with PCR- amplification of β-actin gene, and the synthesized cDNA was kept at -80^{O}C till further analysis.

Real-time PCR analysis: Quantitative real-time RT-PCR (qRT-PCR) of the target genes: TLR4, IL-10 and the reference gene β-actin were performed in LightCycler®480 II-real time PCR detection system (Roche, Germany). Amplifications were carried out in 10 µl reaction volume, containing 1.0 µl of cDNA, 0.25 µl of FW and RV primers (2.5 µM each; table-1), 5 µl of 2X lightCycler®480 SYBR Green I master mix (Roche, Germany) and 3.5 µl of PCR grade H_2O. The qRT-PCR was performed in triplicate wells under following conditions: initial denaturation at 95ºC for 10 min followed by 45 cycles of 94ºC/10 s, 55ºC/10 s and 72ºC/10 s. The reaction carried out without cDNA was used as negative control. The PCR efficiencies were determined by analyzing the serial dilutions of cDNA, and efficiencies were almost 100% which allowed the use of $2^{-\Delta\Delta CT}$ method for calculation of relative gene expression of the target genes TLR4 and IL-10 with that of reference gene, β-actin[20]. The correct size and single band amplification was confirmed by analyzing 8 µl of the real time PCR products in ethidium bromide-stained 2% agarose gel. The relative expression ratios were obtained by normalizing expression of the target gene, as determined by mean crossing point (Cp) deviation by that of a non-regulated reference gene, β-actin following $2^{-\Delta\Delta CT}$ method. The data obtained from qRT-PCR analysis was expressed as mean of three individual experiments ± standard error (s.e.), and the significant difference between the control and treated groups at each time point was determined by the Student's *t*-test using Microsoft Excel 2010 with $p < 0.05$ as significance level.

Results and Discussion

Identification of TLR4 gene in mrigal: The partial cDNA of TLR4 gene in mrigal was cloned from gill. The heterologous PCR primer set obtained from the conserved domain of TLR4 among zebrafish, mouse and chicken generated 204 bp fragment. Cloning, sequencing and BLAST search confirmed it as TLR4 gene in mrigal, and was submitted to the GenBank with the accession number: GU248419.

Till date, TLR4 gene was only identified in zebrafish and grass carp[12-13, 21]. The identification of TLR4 gene in mrigal indicates the wide distribution of TLR4 among various fish species.

TLR4 gene expression during developmental stages: In mrigal, embryonic developmental profile of TLR4 gene expression was analyzed by quantitative real-time PCR (qRT-PCR) assay and the expression at various stages was represented as relative fold changes from the 0h, chosen as calibrator (1). As shown in figure-1A, constitutive expression of TLR4 gene was detected throughout the embryonic developmental stages and in hatchlings but the expression levels in other developmental stages were less than the fertilized eggs (0h). These results were indicative to the protective role of TLR4 during early stages of development in pathogenically hostile aquatic environment.

In mrigal embryo, expression of TLR2 and TLR5 were previously been reported suggesting the innate immune mechanism of embryos in recognizing LTA, PGN and flagellin[22-23]. In addition to these TLR genes, detection of TLR4 expression indicated the ability of the mrigal embryos to recognize LPS. Our observation in mrigal is in accordance with the similar observation of TLR4 gene expression in grass carp embryo[24].

Tissue distribution of TLR4: Tissue specific expression of TLR4 gene in muscle, eye, blood, intestine, brain, heart, kidney, liver, spleen, gill and skin was evaluated by qRT-PCR assay and the expression of TLR4 gene in various organs/tissues was represented as relative fold changes from the lowest TLR4 expressing tissue, muscle, chosen as calibrator (1). As shown in figure-1B, TLR4 expression was at detectable levels in all examined tissues but the level of expression varied among the tissues. Lowest TLR4 expression was in muscle and the highest was in skin.

The TLR4 gene expression in various organs/tissues was previously been reported in zebrafish and grass carp[12-13, 24]. In mrigal, almost all tested tissues expressed TLR4, but the pattern of TLR4 gene expression significantly differs from zebrafish and grass carp. Wide expression of TLR4 in various organs

indicated the important role of TLR4 in sensing pathogenic invasion and induction of innate immunity.

Modulation of TLR4 expression by LPS: In higher vertebrates, LPS is a well-known ligand of TLR4. To investigate LPS-mediated TLR4 response in fish, LPS was i.v. injected, and TLR4 expression in various tissues of mrigal was analyzed after 4 h by qRT-PCR assay. As shown in figure-2A, TLR4 expression was significantly ($p < 0.05$) up-regulated in all the tested tissues of LPS-treated fish as compared to control fish and the highest induction of TLR4 was observed in the intestine (~ 32 fold). In gill, liver, kidney and blood, the magnitude of TLR4 induction was ~ 1.9, 1.2, 4.7 and 1.4 fold respectively as compared to their control.

These data indicated LPS sensitivity of TLR4 in mrigal, and was in agreement with the previous observation in grass carp[13, 24]. However, the observation in grass carp and mrigal significantly differ from the previous observation of TLR4 in zebrafish showing tolerance to LPS[11, 25].

Modulation of TLR4 gene expression in *A. hydrophila* infection: To investigate the *in-vivo* response of TLR4 gene in bacterial infection, mrigal fingerlings were i.p. injected with *A. hydrophila*, a Gram-negative bacteria, and TLR4 expression was monitored in gill, liver, kidney, intestine and blood at 6, 12 and 24 h post treatment (p.t.) by qRT-PCR. In *A. hydrophila* infected fish, modulation of TLR4 gene expression was observed in all tested organs at various time course (figure-2B). In the infected fish intestine, most significant ($p < 0.05$) and gradual induction of TLR4 gene expression was observed at 6, 12 and 24 h p.t. with the enhanced induction of ~ 2.6, 4 and 4.3 fold respectively. In gill, TLR4 up-regulation (~ 3 fold) was observed only after 24 h. In blood, a quick induction of TLR4 was observed at 6 h p.t. but it was down regulated at 12 and 24 h p.t. In the infected fish liver and kidney, TLR4 expression was down-regulated as compared to control fish.

A. hydrophila is a Gram negative bacterium, which contains high concentration of LPS. Therefore induction of TLR4 in *A. hydrophila* infection in mrigal and grass carp was expected[13]. These data together suggested a critical role of TLR4 in sensing *A. hydrophila* infection in various fish species.

Modulation of IL-10 expression in LPS treatment and *A. hydrophila* infection: In LPS- treated fish tissues, IL-10 was significantly ($p < 0.05$) up-regulated in all the examined tissues as compared to control. The highest induction was observed in treated fish liver (~ 260 fold) at 4 h post LPS-treatment. In gill, kidney, intestine and blood, the induction level of IL-10 was ~11.8, 81, 125.8 and 21.7 fold respectively (figure-3A).

In *A. hydrophila* infected fish, most significant ($p < 0.05$) induction of IL-10 was observed in the liver and intestine. At 6, 12 and 24 h post infection, IL-10 induction was ~ 9, 2 and 4 fold respectively in liver, and ~3, 3.2 and 2.5 fold respectively in the intestine as compared to control. In the infected fish kidney, there was 2.9 fold induction at 12 h and 3.5 fold induction at 24 h post infection. In blood, significant up-regulation of IL-10 (~3.6 fold) was only at 6 h post infection. IL-10 expression in gill was down-regulated in treated fish at 6 h and 12 h as compared to control fish (figure-3B).

TLR4 activates signaling through MyD88-dependent pathway. In this pathway, activation of TLR4 leads to NF-κB phosphorylation, and induction of several cytokines and chemokine. In human, mouse and other vertebrates, IL-10 was induced by TLR4 through NF-κB and functioned as an anti-inflammatory cytokine[26]. In the Indian major carp, catla (*Catla catla*), IL-10 was shown to be induced through NF-κB activation[27]. As catla and mrigal are very closely related fish species, IL-10 induction by LPS through TLR4 signaling is also expected to be through NF-κB-signaling. Further works are required to confirm this.

Conclusion

This report describes the identification of TLR4 in the Indian major carp mrigal, a very important and extensively cultured freshwater fish species in the Indian subcontinent. LPS treatment and *A. hydrophila* infection activated TLR4 -signaling resulting in IL-10 induction. This is the first report of TLR4 in the Indian major carps.

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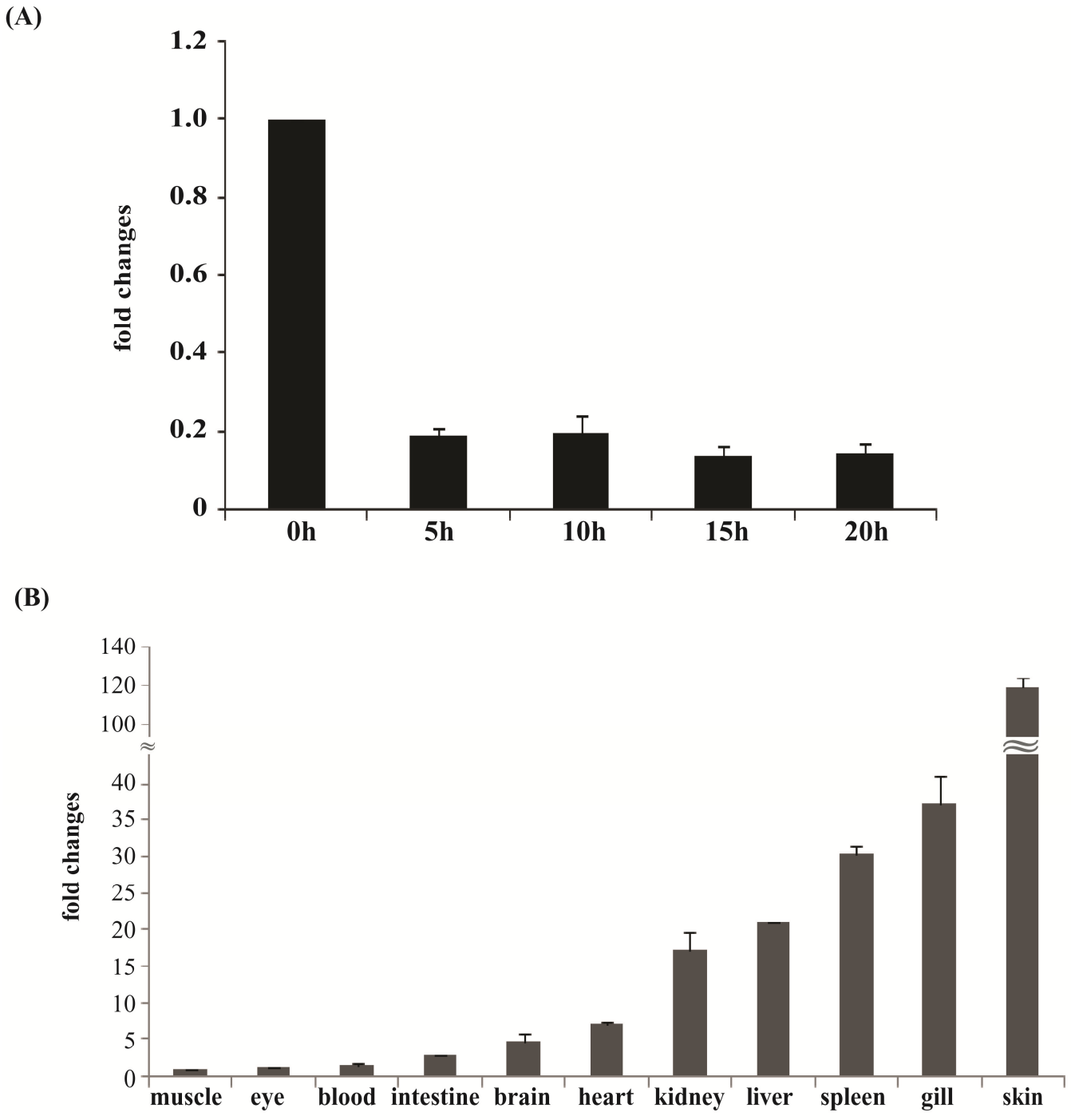

Figure-1
In vivo **expression profile of TLR4 gene in mrigal, (A) TLR4 gene expression at various developmental stages, (B) Tissue specific expression of TLR4 gene in various organs/tissues**

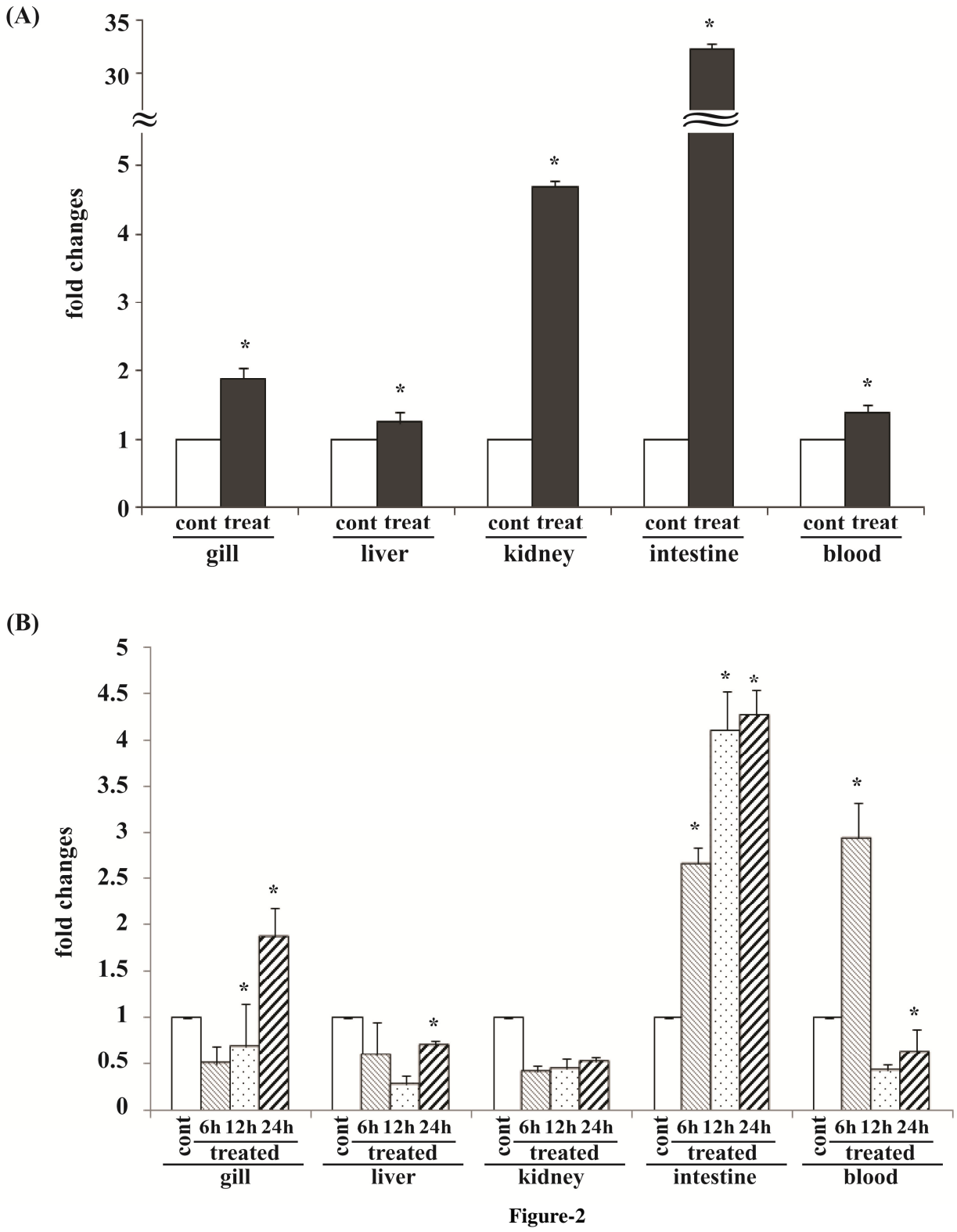

Figure-2

Modulation of TLR4 gene expression following ligand stimulation and bacterial infection, (A) LPS stimulation, (B) *A. hydrophila* infection. Significant difference ($p < 0.05$) between the control and treated group was indicated with asterisks (*)

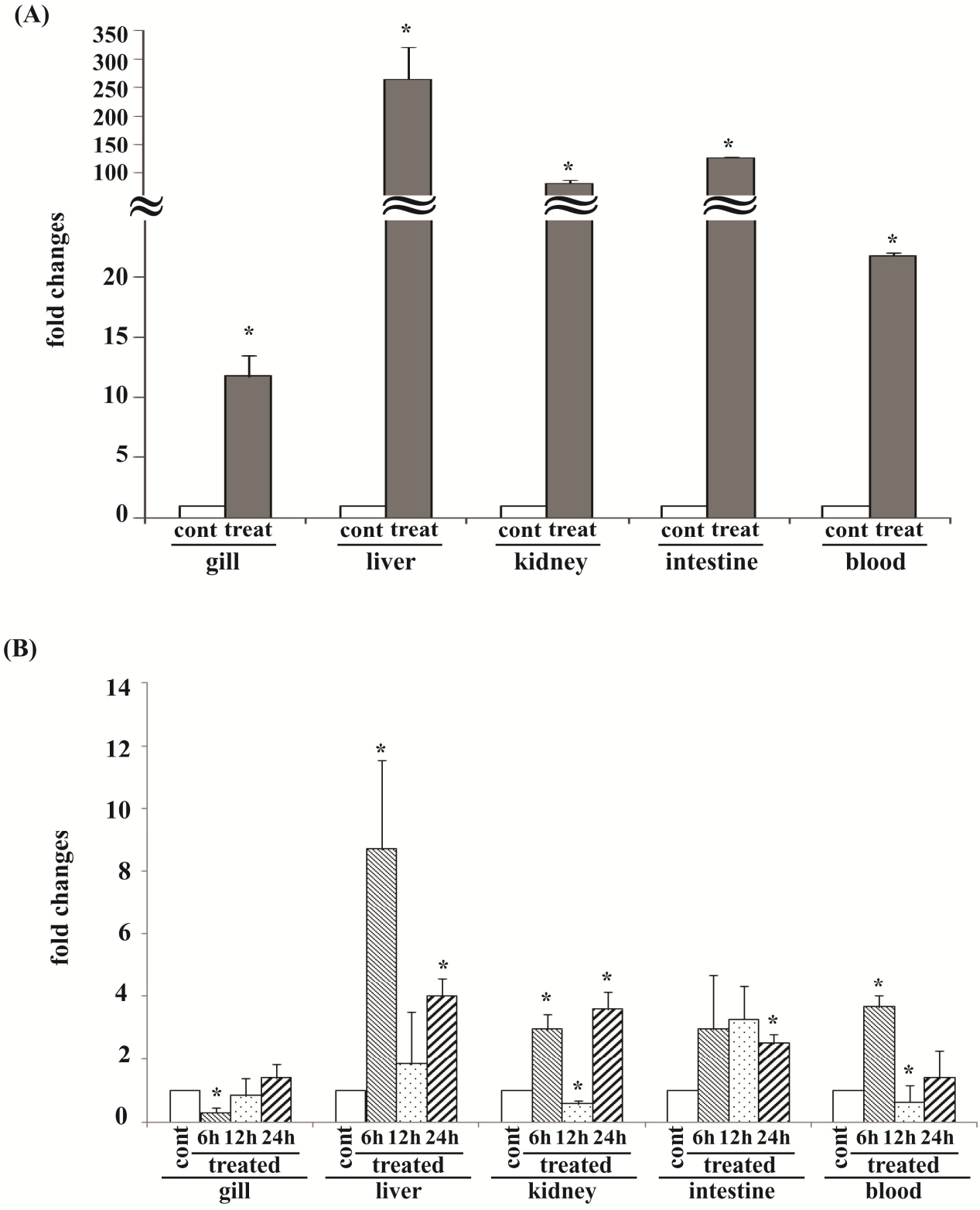

Figure-3

Modulation of IL-10 gene expression following ligand stimulation and bacterial infection, (A) LPS stimulation, (B) *A. hydrophila* infection. Significant difference ($p < 0.05$) between the control and treated group was indicated with asterisks (*)

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