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Inflammatory cytokines responses of common carp, *Cyprinus carpio*, leucocytes *in vitro* treated by immunostimulants

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Abstract

Cytokines are important regulators of the immune system, and identifying fish cytokines has potential applications for the development of vaccines and/or immunostimulants application in fisheries. In order to understand the immune-related genes triggered by immunostimulants derived from pathogens, we investigated the effects of agonists (lipopolysaccharide (LPS), Poly I:C, and imiquimod) of three Toll-like receptor (TLR)—TLR4, TLR3, and TLR7, respectively—on the expression level of 10 cytokine genes—interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), CXC-chemokine, interleukin-10 (IL-10), type1 interferon (1INF), IFN-γ1, IFN-γ2, Mx protein, and interleukin-12 (IL-12p35 and IL-12 p40)—in the head kidney leukocytes of common carp (*Cyprinus carpio* L.). All cytokine genes tested, except for type-1 IFN, were significantly up-regulated after LPS treatment. Poly I:C and imiquimod treatment resulted in striking up-regulation of most of the genes examined, particularly for the interferon genes as compared with the control groups. These results clearly demonstrate that TLRs agonists elicit the signaling pathways for cytokines production and initiation of innate immune responses in common carp. The development of strategies to control the pathogen load and of immune-prophylactic measures must be addressed further to realize the economic potential of fish production.

Keywords: LPS, Poly I:C, Imiquimod, Carp, Cytokines.

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Introduction

Cytokines are small glycoproteins involved in extracellular signaling and play a significant role in balancing host immune responses. Based on their structural features, cytokines have been grouped into several families, including IL-1, -2, -6, -12, -10, -17, 1TNFs, Tumor necrosis factors (TNFs), transforming growth factors, and chemokines (Vilcek *et al.*, 2003). These molecules regulate local and systemic immune inflammatory and regulatory events. The differentiation of Th cells into Th1–Th2 cells is important as hosts mount effective immune responses. The differentiation and balance between Th1–Th2 type response is predominately mediated by the respective cytokines. Previously, identification of fish cytokines was based mainly on EST- and PCR-mediated homology cloning using degenerate PCR primers (Savan and Sakai, 2006).

Several fish cytokine genes have been isolated and characterized in recent years, and researchers have used mRNA expression as a tool for measuring immune responses (genomic database of zebrafish and fugu). Although not all genes discovered by homology cloning may encode for proteins with the same function as the query genes, it appears that fish have most of the cytokines relevant to responses to bacteria, viruses, inflammation, or cell proliferation and chemo-attraction. In particular, pro-inflammatory cytokines, including IL-1β, TNF-α, IL-8, IL-12, INFs, and IL-10 are commonly used immune-regulatory genes in fish (Castillo *et al.*, 2009, Tanekhy, 2014).

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and utilize conserved signaling pathways to activate pro-inflammatory cytokines and type1 interferons (1INFs). PAMPs are essential for microbial survival and are shared by large groups of microorganisms. They are recognized by Toll-like antigen receptors called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000; Tanekhy, 2014). Recognizing conserved structures shared by large groups of microorganisms is advantageous because it allows innate immunity to recognize many different microorganisms with a limited number of host PRRs (Akira *et al.*, 2006). After recognizing PAMPs, TLRs activate intracellular signaling pathways via a set of intracellular TLR-domain-containing adaptors. The ligands for mammalian TLRs have been reviewed, and there is an evidence for teleost TLRs. To date, a total of more than 17 teleosts TLRs have been reported in more than a dozen different fish species (Rebl *et al.*, 2010). The essential structures and functions of TLRs and their related molecules are highly conserved between teleost and mammals. The TLR system for sensing microorganisms is diverse and important in teleosts too, and the fish system has similarities with the mammalian system. In addition, the functional importance of fish-specific

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TLRs is becoming clear recently (Matsuo *et al*., 2008). Elucidation of TLR-mediated host-pathogen interaction in teleosts may lead to an effective alternative strategy for disease control in fish farming.

TLR signaling pathways are regulated by various combinations of adaptor molecules. It is, therefore, generally agreed that multiple complex mechanisms in the TLR signal pathway are tightly controlled to elicit innate immune responses against particular pathogens (Akira *et al*., 2006; Tanekhy, 2014).

Furthermore, recent studies suggest the importance of TLR-controlled innate immune responses in orchestrating subsequent acquired immune responses (Iwasaki and Medzhitov, 2004). Hence, studies on TLR mechanisms are important for developing potent and specific immunotherapeutic methods against infectious disease.

The present study was carried out to investigate the effect of lipopolysaccharide (LPS), poly I:C, and imiquimod—ligands for TLRs—on cytokine genes expression in common carp, *C. carpio* L, in order to understand individual TLRs systems and their corresponding cytokines in teleosts.

Materials and methods

Fish

Common carp, *Cyprinus carpio* L, (mean weight 120 g, n=30) were obtained from Sasaki fishery, Miyazaki, Japan. The fish were maintained in outdoor tanks with running fresh water at 16°C; 50% of the water was exchanged weekly to maintain water quality for 2 weeks. Animals were fed with commercial diet twice daily.

Treatment with LPS, Poly I:C and imiquimod

Fish were randomly selected, and head kidney (HK) cells were isolated as previously described (Braun-Nesje *et al*., 1982). HK cells were isolated through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1% streptomycin/penicillin (S/P, Gibco, USA), 0.2% heparin (Sigma, USA) and 10% carp serum (CS). HK cells (1×10^7 cells/mL) were stimulated by incubation with 10 µg/mL (preliminary determined) of LPS (Sigma, USA), Poly I:C (Sigma, USA), or imiquimod (Sigma, USA) for 0, 1, 4, 8, 12, 24, or 48 h. Tissue samples were preserved in ISOGEN (Nippon Gene, Toyama, Japan) and stored at -80 °C.

Expression analysis of cytokine genes

Analysis of the IL-1β, TNF-α, CXC-chemokine, IL-10, IL-12p35, and IL-12 p40, type1 INF, Mx protein, and IFN-γ1, IFN-γ2 genes was performed on HK cells at 0, 1, 4, 8, 12, 24, and 48 h after LPS, poly I:C, or imiquimod treatments. Total RNA was isolated from HK cells using ISOGEN according to the manufacturer's instructions. Polyadenylated RNA was purified using a quick prep micro mRNA kit (GE Healthcare, Sweden). All RNA isolates had an OD_{260}: OD_{280} between 1.8 and 2.0, indicating clean RNA isolates. The

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RNA quality was also checked by 1.0% agarose gel electrophoresis, stained with 1 ug mL^{-1} ethidium bromide. cDNA synthesis was performed using ReverTra Dash (Tokyo, Japan). All PCR reactions were performed as previously described (Kono *et al*., 2004; Tanekhy *et al*., 2010). Briefly, 1 µL cDNA was mixed with 5 µL buffer, 5 µL dNTPs (10 µM each of dNTP), 0.5 µL *Taq* polymerase (5 units µL^{-1}), 5 µL (5 µM) each of gene-specific primer, and 27.5 µL water. Accession numbers of the cytokine genes and the β-actin control gene along with the primers used for amplification are shown in Table 1. The optimum amplification conditions for each reaction and the expected product sizes are shown in Table 2. PCR products were electrophoresed on 1.5% agarose gel. After determination of the optimum cycle number, three replicates of each PCR were conducted. The relative expression levels for carp cytokine genes mRNAs were determined using the carp β-actin gene as internal control.

The cytokine/β-actin ratio was determined by densitometry. The photostimulated luminescence values were measured using Science Lab99 Image Gauge software (Fujifilm, Tokyo, Japan).

Table 1: Primer sequences of common carp and their accession numbers in gene bank.

Primers`	Sequece (5'-3')	Nucleotide No.	Access. No.	Information regarding primers
RT-PCR analysis				
CXC-chemokine F	GTGTGAACATGGTTCCTCCA	20	AB082985	Expression analysis
CXC-chemokine R	GGATTGAAGCATTTCTGCTCT	21		Expression analysis
INF-α F	TGCATATGGCTCGGCCAATA	20	AB376667	Expression analysis
INF-α R	GTCAAGACAAGAAACCTCACC	21		Expression analysis
IL-1β F	GGAGAATGTGATCGAAGAGC	20	AJ245636	Expression analysis
IL-1β R	GTAGAGGTTGCTGTTGGA	18		Expression analysis
IL-10 F	TGATGACATGGAACCATTACTGG	23	AB110780	Expression analysis
IL-10 R	CACCTTTTTCCTTCATCTTTTCA	23		Expression analysis
IL-12p35 F	TGCTTCTCTGTCTCTGTGATGGA	23	AJ580354	Expression analysis
IL-12p35 R	CACAGCTGCAGTCGTTCTTGA	21		Expression analysis
IL-12p40 F	GAGCGCATCAACCTGACCAT	20	AJ621425	Expression analysis
IL-12p40 R	AGGATCGTGGATAGTAGCCTCTAC	24		Expression analysis
TNF-α F	GCTGTCTGCTTCACGCTC	18	AJ311800	Expression analysis
TNF-α R	AAAGCCTGGTCCTGGTTC	18		Expression analysis
INFγ1 F	GTCGCTGCTGCTTGATAGAA	20	AM261214	Expression analysis
INFγ1 R	CTGAAGCTCCCTCCATACTT	20		Expression analysis
INFγ2 F	GAGGAACCTGAGCAGAATCT	20	AM168523	Expression analysis
INFγ2 R	CCTTGATCGCCCATAGTGTT	20		Expression analysis
Mx F	GTGCAGAGGTCAGCAAGACA	20	EF635410	Expression analysis
Mx R	CCTGTGGCAGTGTTTTAGCA	20		Expression analysis
B-actin F	ACCTCATGAAGATCCTGACC	20	M24113	Expression analysis (control)
B-actin R	TGCTAATCCACATCTGCTGG	20		Expression analysis (control)

Statistical analysis

The PCR results in the treated and control groups were analyzed by one-way analysis of variance (ANOVA) by using SPSS software 14. A multiple comparison method (Turkey) has been used.

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Table 2: Conditions for PCR for the cytokine genes in carp.

Cytokine genes	Product size bp	Cycling conditions		Cycle No.
		Annealing T	Extension T	
CXC-chemokine	123	59°C/30 s	72°C/45 s	30
INF-α	804	61°C/30 s	72°C/75 s	35
IL-1β	280	61°C/30 s	72°C/60 s	30
IL-10	284	60°C/30 s	72°C/60 s	30
IL-12p35	86	61°C/30 s	72°C/45 s	30
IL-12p40	150	61°C/30 s	72°C/45 s	30
TNF-α	188	58°C/30 s	72°C/60 s	30
INFγ1	168	60°C/30 s	72°C/60 s	32
INFγ2	202	60°C/30 s	72°C/60 s	32
Mx	187	60°C/30 s	72°C/60 s	32
B-actin	312	60°C/30 s	72°C/45 s	24

Results

The effect of LPS on the expression of carp cytokine genes

In carp HK cells treated with LPS, cytokine gene expression was up-regulated (Fig. 1). IL-1β was significantly up-regulated at 4, 8, 12, and 24 h post treatment; TNF-α was significantly up-regulated at 1, 4, 8, 12 and 24 h post treatment; CXC-chemokine showed variable expression levels upon treatment with LPS, and it was significantly up-regulated 24 and 48 h after LPS treatment; IL-10 gene expression was significantly up-regulated 8 h after LPS treatment (Fig. 1a). IL-12p35 gene expression showed significant up-regulation 8, 12, 24, and 48 h after LPS treatment, and IL-12p40 gene expression exhibited significant up-regulation 8, 12, and 24 h after LPS treatment (Fig. 1b). Type-1 IFN and Mx protein gene were not expressed after LPS treatment (Fig.1c). INF-γ1 gene expression was significantly up-regulated after 8 and 12 h of LPS treatment while INF-γ2 gene expression showed significant up-regulation only after 8, 12, 24 and 48 h of LPS treatment (Fig. 1d) as compared with control group.

The effect of poly I:C on the expression of carp cytokine genes

IL-1β was significantly up-regulated 1 h after treatment with poly I:C treatment with a peak of expression at 8 h post-treatment. TNF-α expression was up-regulated after poly I:C treatment; however the increase in expression at 12 h was not statistically significant. CXC was significantly up-regulated 8, 12 and 24 h after treatment. IL-10 was significantly up-regulated within 1 h of treatment and 4, 8, and 48 h after poly I:C treatment (Fig. 2a).

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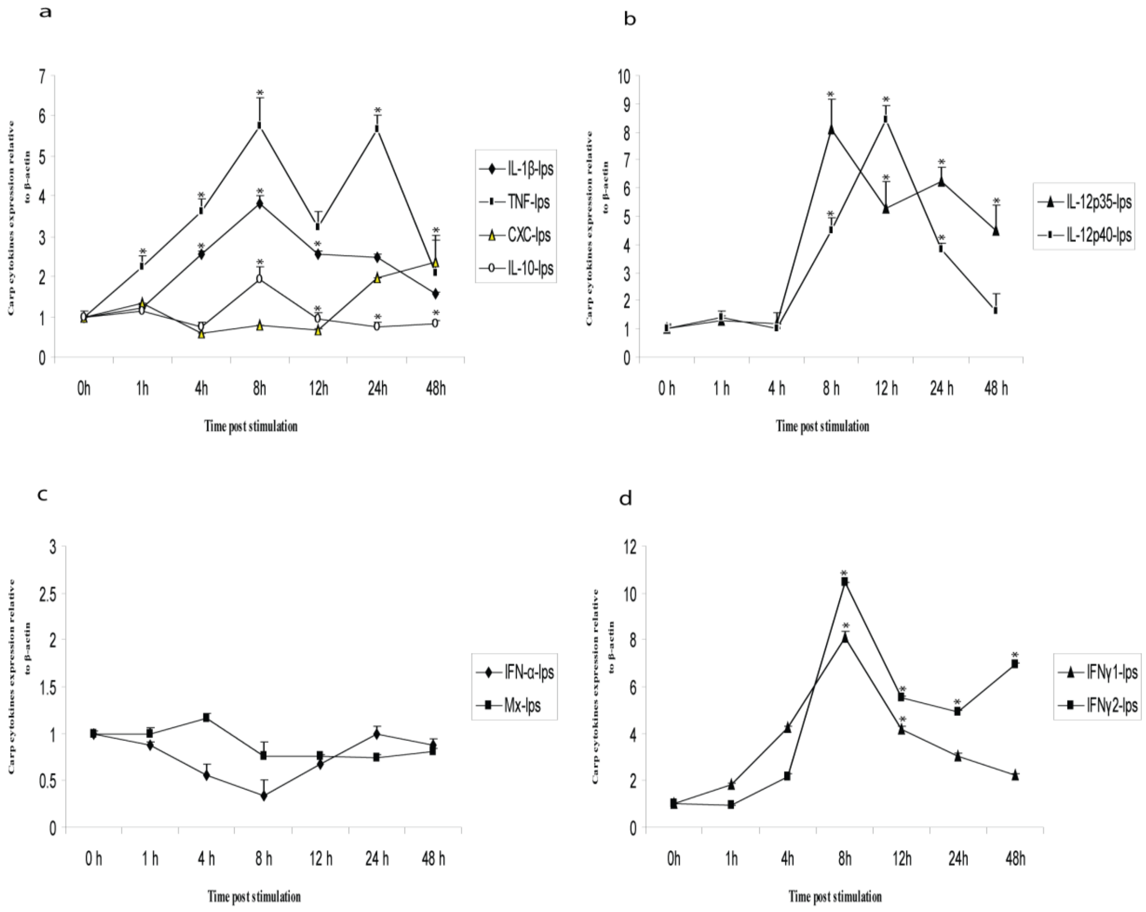

Figure1: Semi-quantitative PCR analysis of the carp cytokine genes in head kidney cells stimulated with LPS. PCR was performed using primers and probes specific for the carp cytokine genes and β-actin gene with cDNA synthesized from head kidney cells incubated with 10 μg ml^{-1} of LPS for 0 (control), 1, 4, 8, 12, 24 and 48 h. The expression values were normalized against the expression of β-actin gene. Data are presented as mean+SD of triplicate samples. a, is for the pro-inflammatory cytokines, b for IL-12, c for type 1 INF and its marker and d for type 2 INFs. * *p*<0.05, compared to the control.

IL-12p35 was significantly up-regulated 8, 24, and 48 h post-treatment; while IL-112p40 was significantly up-regulated 1, 8, 24, and 48 h post treatment (Fig. 2b). Significant up-regulation of Type-1 INF and its marker (Mx protein) was detected within 1 h of poly I:C treatment and the up-regulation persisted for 48 h except for a transient decrease in expression at 12 h (Fig. 2c). INF-γ1 gene expression was up-regulated within 1 h after Poly I:C treatment and up-regulation persisted for 48 h except for a transient decrease in expression at 12 h; INF-γ2 gene expression was significantly up-regulated by 4 h after poly I:C treatment and up-regulation persisted for 48 h except that 12 h after poly I:C treatment expression levels seemed to be transiently down-regulated (Fig. 2d) as compared with control group.

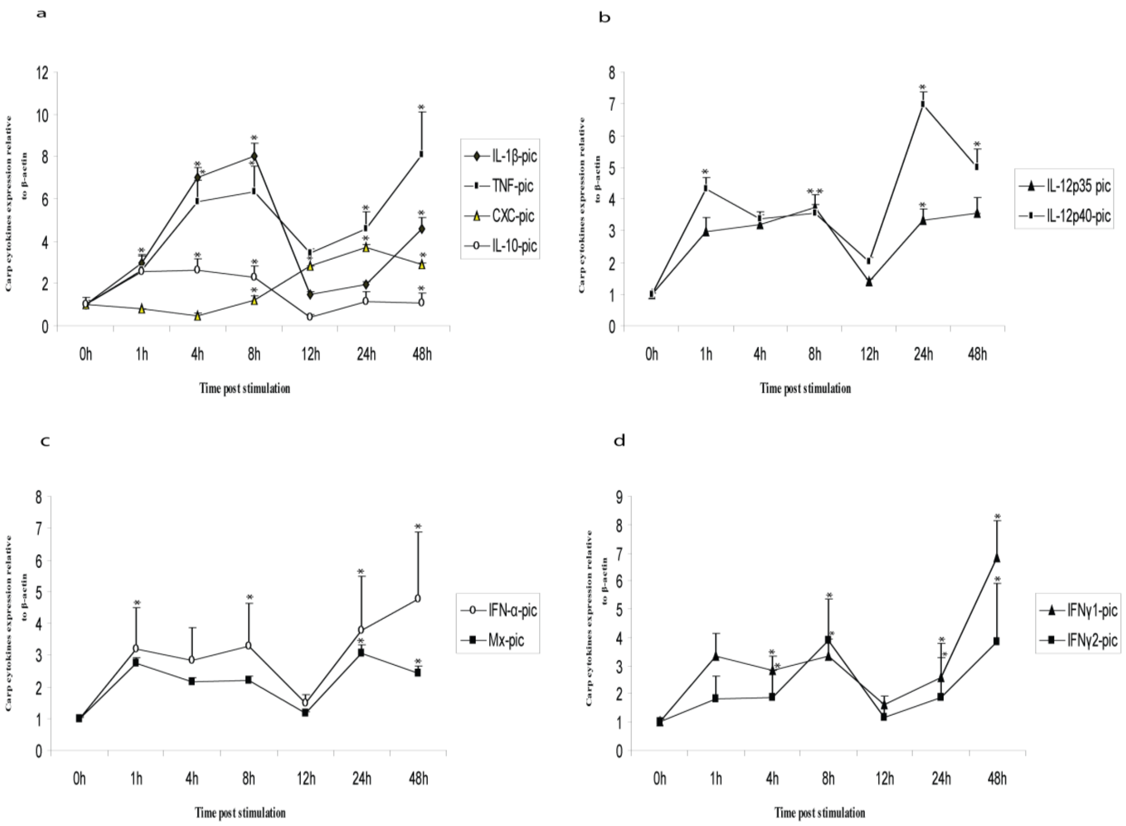

Figure 2: Semi-quantitative PCR analysis of the carp cytokine genes in head kidney cells stimulated with poly I:C. PCR was performed using primers and probes specific for the carp cytokine genes and β-actin gene with cDNA synthesized from head kidney cells incubated with 10 μg ml^{-1} of poly I:C for 0 (control), 1, 4, 8, 12, 24 and 48 h. The expression values were normalized against the expression of β-actin gene Data are presented as mean+SD of triplicate samples. a, is for the pro-inflammatory cytokines, b for IL-12, c for type 1 INF and its marker and d for type 2 INFs. * $p<0.05$, compared to the control.

The effect of imiquimod on the expression of carp cytokine genes

All 10 cytokine genes experienced some up-regulation after imiquimod treatment (Fig. 3). IL-1β showed significant up-regulation at 1, 4, 8, 24, and 48 h; while TNF-α expression showed significant up-regulation at 4, 12, 24, and 48 h post treatment. CXC-chemokine expression levels were significantly up-regulated at 12, 24, and 48 h post-treatment. IL-10 showed significant up-regulation 1, 24 and 48 h after imiquimod treatment (Fig. 3a). IL12p35 showed significant up-regulation at 1 and 24 h; while IL-12p40 was significantly up-regulated at 4 and 8 h after imiquimod treatment (Fig. 3b). Upon imiquimod treatment, type-1 INF was significantly up-regulated at 4, 8, and 48 h post-treatment; while Mx protein showed significant up-regulation at 8 h post treatment (Fig. 3c). INF-γ1 gene expression exhibited significant up-regulation at 4 and 8 h post treatment, and there was significant up-regulation of the INF-γ2 gene at 4, 8, and 48 h after treatment with imiquimod (Fig. 3d) as compared with control group.

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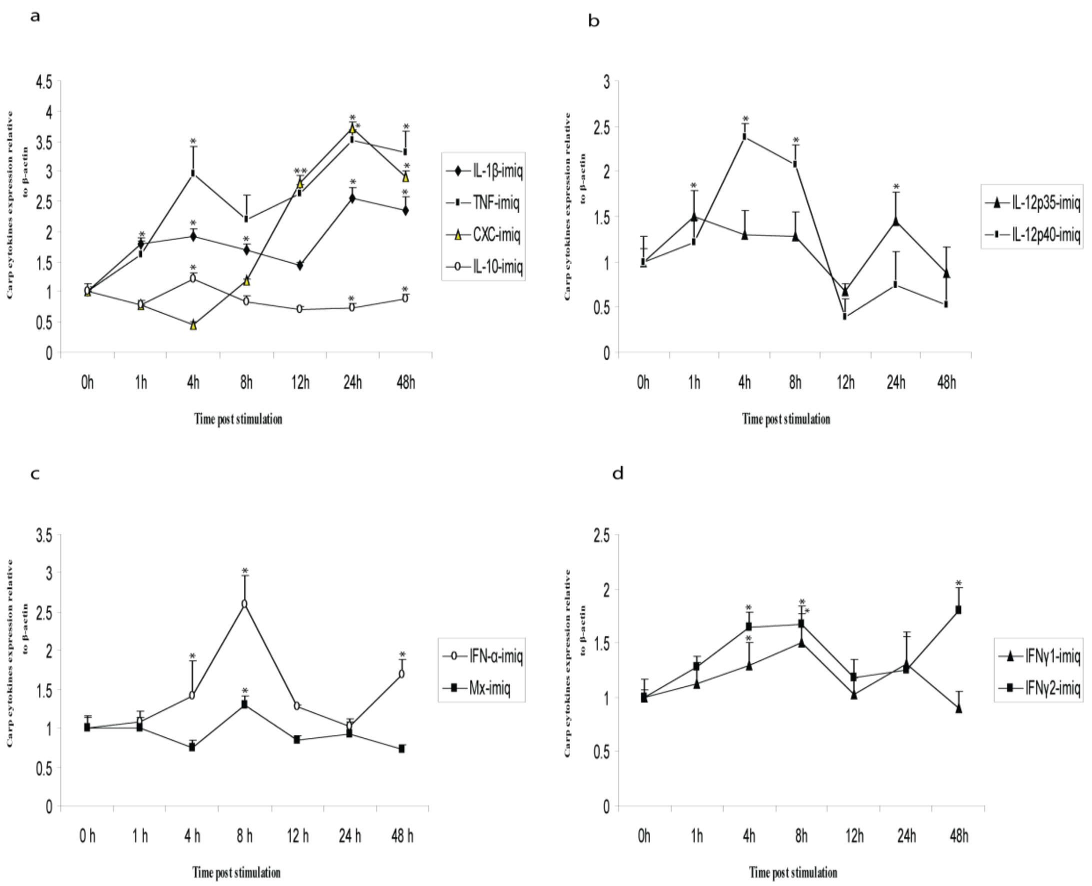

Figure 3: Semi-quantitative PCR analysis of the carp cytokine genes in head kidney cells stimulated with imiquimod. PCR was performed using primers and probes specific for the carp cytokine genes and β-actin gene with cDNA synthesized from head kidney cells incubated with 10 μg ml^{-1} of imiquimod for 0 (control), 1, 4, 8, 12, 24 and 48 h. The expression values were normalized against the expression of β-actin gene Data are presented as mean+SD of triplicate samples. a, is for the pro-inflammatory cytokines, b for IL-12, c for type 1 INF and its marker and d for type 2 INFs. * *p*<0.05, compared to the control.

Discussion

LPS is an integral component of the outer membrane of Gram-negative bacteria and has been used in experimental systems for several decades as a potent immunostimulant. Chemically, LPS includes a lipid domain, lipid A (LA), which is considered to be the endotoxic center of LPS (Rietschel *et al*., 1996). The LPS complex is recognized by TLR4, and the interaction results in cell activation.

The lack of a TLR4 ortholog in some fish species and the lack of the essential co-stimulatory molecules for LPS activation via TLR4 (i.e., myeloid differentiation protein 2 (MD-2) and CD14) in all available fish genomes and expressed sequence tag databases led to hypothesize that the mechanism of LPS recognition in fish may be different from that of mammals (Sepulcre *et al*., 2009). Constitutive expression of pro-inflammatory cytokines (IL-1β, TNF-α, and CXC-chemokine) genes in carp HK cells was observed. These results support previous findings that LPS enhanced pro-inflammatory cytokine expression (Savan and Sakai, 2006; Tanekhy *et al.,* 2010). In this study, significant up-regulation of CXC-chemokine by LPS was delayed to 12 h post-treatment. In contrast, a previous report suggested that CXC-chemokine was immediately up-regulated after LPS treatment (Savan *et al*., 2003). This up-regulation may be due to early up-regulation of IL-10 which behaves as a pro-inflammatory agent in the early infection (Inoue *et al.*, 2005). The early up-regulation of IL-10 gene in carp HK leukocytes may be due to IL-10’s function as a pro-inflammatory cytokine in most teleosts in the early stage of infection (Inoue *et al.,* 2005; Tanekhy *et al*., 2009). The increased expression level of IL-12p35 and IL-12p40 genes together with the up-regulation of TNF-α, IL-12 upon stimulation of carp HK leukocytes by LPS may stimulate the production of IFN-γ and TNF-α from T and NK cells in fish as it does in mammals (Wang *et al*., 2000). The increased levels of gene expression observed in this study were similar to the obtained results (Huising *et al*., 2006) after LPS treatment in which pro-inflammatory cytokine expression levels (IL-1β, TNF-α, IL-12 (subunits p35 and p40) increased considerably with a concomitant increase of nitric oxide (via inducible nitric oxide synthase) and toxic oxygen and nitrogen radicals directed to killing the invading pathogens. The increased expression level of INF-γ genes after LPS treatment is similar to previous findings; although NK cells do not respond to LPS treatment (Stolte *et al*., 2008). In some cases, HK phagocytes increase IFN-γ1 expression after LPS stimulation, and a lingering effect of B-lymphocytes may be present, as phagocyte fractions that result from density separation always contain some lymphocytes (Kemenade *et al*., 1994). These observations were added to the complexity of models of fish immune responses and may reveal new immune mechanisms. In fact, the expression of carp IFN genes may be regulated by TLR-dependant and TLR-independent pathways. This model suggests that alternative signaling-receptors, in particular beta-2 integrins, may play a primary role in the activation of teleost leukocytes by LPS (Iliev *et al*., 2005; Tanekhy, 2014).

Poly I:C is known to interact with TLR3, which is expressed in the intracellular compartments of B-cells and dendritic cells. Poly I:C is structurally similar to dsRNA and poly I:C is a "natural" activator of TLR3. Thus, Poly I:C can be considered a synthetic analog of dsRNA and is a common tool for scientific research on the immune system. The antiviral activity generated after ligand binding and intracellular signaling has been attributed to the production of type-1 INF and its marker, Mx proteins (Jensen and Robertsen, 2000). Human natural killer cells (NK cells) have been reported to express TLR3, up-regulate TLR3 mRNA, and increase cytotoxic activity following poly I:C stimulation, and NK cells produced higher amounts of IL-6, IL-8, and IFN-γ after TLR3 stimulation (Bricknell and Dalmo, 2005).

Our findings demonstrated that poly I:C significantly increased pro-inflammatory cytokines—IL-1β, TNF-α, and CXC-chemokine—expression in carp HK cells. TNF-α expression in carp was previously studied, and it was up-regulated after Astragalus polysaccharides (APS) stimulation (Bricknell and Dalmo, 2005) and CpG oligodeoxynucleotides (CPG-ODN) (Yuan *et al*., 2008) as a pro-inflammatory cytokines. CXC-chemokine was significantly up-regulated in response to viral particles (either ssRNA or dsRNA) to induce the migration of monocytes and other cell types to the site of inflammation. The early up-regulation of the IL-10 gene in carp HK leukocytes functioned as a pro-inflammatory cytokine in fish during the early stage of the inflammatory response. Up-regulation of the IL-12p35 and IL-12p40 genes upon stimulation of carp HK leukocytes by poly I:C may enhance the cytotoxic effects of both NK cells and CD8 for eliminating of intracellular pathogens.

Type-1 IFN was up-regulated upon stimulation of carp HK leukocytes using Poly I:C. The stronger relative IFN-α response to poly I:C in HK leukocytes is thus expected because of the poly I:C receptor MDA5 is expressed in most nucleated cells, which will outnumber cells like pDCs. This observation supports a previous finding that type-1 IFN is significantly expressed upon stimulation of carp HK leukocytes by poly I:C treatment (Kitao *et al*., 2009; Tanekhy *et al*., 2010).

As a sequellae of IFN induction, Mx protein was up-regulated after *in vitro* treating carp HK leukocytes with poly I:C, and induction of Mx protein in carp are most likely via type-1 INF. Following the induction of INF responses by injection of poly I:C in Atlantic salmon parr, Mx protein is produced in tissues and blood leukocytes and present in plasma for about 6 weeks. Therefore, it is evident that teleost TLR3 is able to sense dsRNA (Das *et al*., 2009).

In this study, IFNγ1 and IFNγ2 genes were up-regulated after poly I:C treatment; however, it was reported that Poly I:C did not reproducibly induce IFN-γ1 or IFN-γ2 gene expression in common carp (Stolte *et al*., 2008). Despite the apparently different expression profiles and functions of these two genes, they utilize similar pathways. In this study, the expression profile of both INF-γ1 and INF-γ2 were nearly similar in carp HK cells after treatment with each TLR ligands. Although it was found that INFγ is up-regulated following poly I:C treatment in salmon (Grayfer and Belosevic, 2009).

The response of HK carp cells to poly I:C in this study were similar to the results reported from clonal catfish lymphatic and fibroblastic cell lines treated with poly I:C, which resulted in the expression of type1 INF and the subsequent induction of ISGs (interferon-stimulated genes) as Mx, ISG15 and CXCL10 (Milovanovic *et al*., 2009). Poly I:C activates the IFN-promoter stimulator-1 (IPS-1) and Toll/IL-1R domain containing adaptors inducing IFN-β (TRIF)-dependent pathways in CD8α cDCs, which in turn leads to NK cell activation. This immune response to poly I:C is helpful for protection against viral infection. Therefore, the use of an appropriate TLR ligand as a vaccine adjuvant is a promising approach to improve the protective ability of vaccines (Nishizawa *et al*., 2009).

Imiquimod, a low molecular weight imidazoquinoline, is an immune response modifier, which has potent anti-viral and anti-tumor properties. Imiquimod is a TLR7 ligand (Akira and Hemmi, 2003) that induces NF-kB translocation and production of IFN-α, TNF-α, IL-6, and IL-12 upon binding to TLR7 (Jault *et al*., 2004). The resulting CD4C T cell activation and Th1 immune response are vital for the host's antiviral defense. The natural ligand for TLR7 is currently unknown. TLR7 gene is expressed in many organs and tissues in adult zebrafish, and it is expressed during zebrafish embryonic development (Miller *et al.*, 2008). TLR7 induces a cytokine cascade and enhances the ability of APC to present viral antigens to reactive T lymphocytes, thereby promoting an antigen-specific Th1 cell-mediated immune response (Stanley, 2002).

Upon imiquimod treatment, pro-inflammatory genes expression are enhanced in carp HK leukocytes and quite similar up-regulation are seen after poly I:C treatment. The increases in IL-1β, TNF-α, and CXC in the first 24 h and the up-regulation of IL-10 after 24 h is similar to the response of Atlantic salmon after treatment with imiquimod (Linn, 2007; Tanekhy *et al*., 2010). The up-regulation of Type-1 IFN in HK cells treated with imiquimod is not prominent as that seen after treatment with poly I:C. The difference in induction of IFN by imiquimod and poly I:C may result from a difference in the target’s cell of two stimulants; imiquimod primarily induces IFNs through immune cells whereas poly I:C induces IFNs in most nucleated cells. S-27609 induced much lower levels of IFN than poly I:C in the early time stages (14-48 h) both in the liver and head kidney of salmon (Kileng *et al.*, 2008).

As virus infection can directly induce the expression of Mx protein, Mx expression was up-regulated in response to imiquimod (Ronni *et al*., 1995; Altmann *et al*., 2004). Up-regulation of Mx protein followed INF up-regulation in carp HK cells treated with imiquimod (Kitao *et al*., 2009). Imiquimod seems to be one of the most effective inducers of IFN-γ gene expression (Linn, 2007) as a production of IFN-γ in fish is regulated mainly by the early initial inflammatory responses (Feghali *et al*., 1997). Although salmonid TLR7 is able to discriminate between ssRNA and dsRNA to activate distinct signaling pathways or produce particular cytokines such as the IFN-α genes (Sun *et al*., 2009), this observation was not confirmed in our study, as IFNs were up-regulated after both poly I:C and imiquimod treatment.

In conclusion, TLRs agonists (LPS, Poly I:C, and Imiquimod) have a significant role in activating the immune system in fish. LPS has the ability to enhance cytokine gene expression and is able to attract and prevent bacterial pathogenesis. Although fish are lacking LPS-TLR4 pathway, cytokine network keeps working which indicates that there is another TLR in fish (our next work) instead of TLR4 in other vertebrates. Moreover, poly I:C and imiquimod are able to protect carp from dsRNA and ssRNA viruses. Although it is evident that pathogen-dependent skewing of acquired immune responses is a capacity shared by vertebrates from fish to mammals, it is currently unclear whether the differential responses in lower vertebrates respond to descriptive such as Th1 and Th2 or not. Therefore, further study of the regulation of immunity in direct descendants of early vertebrates may assist us in appreciating the evolutionary significance of the paradigms that shape the field of immunology.

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