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GILL MEDIATES IMMUNE RESPONSES AFTER GRASS CARP REOVIRUS CHALLENGE IN GRASS CARP (*CTENOPHARYNGODON IDELLA*)

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Abstract: Gill plays an important physical barrier role in defending environmental microbes. How are immune responses to endogenous viruses in gill? In the present study, mRNA expressions of 12 antiviral immune-related genes were examined by quantitative real-time RT-PCR (qRT-PCR) in grass carp (*Ctenopharyngodon idella*) gill after grass carp reovirus (GCRV) challenge. The relative values of *CiTLR*3, *CiTLR*7, *CiTLR*22, *CiRIG-I*, *CiMDA*5, *CiLGP*2, *CiNOD*1, *CiNOD*2 and *CiIFN*-I were almost up-regulated at 12h, 24h, 48h and 72h. Additionally, the mRNA expression of *CiIgM* was triggered at 72h. However, relative expressions of *CiMyD*88 and *CiIPS*-1 were down-regulated at 6h, and subsequently increased. To further verify the reliability of viral infection, *VP*4 gene (outer capsid protein of GCRV, segment 6) was checked by RT-PCR amplification. The results indicate that gill serves as an important immune organ, and plays crucial roles in triggering antiviral immune responses in grass carp.

Key words: Immune responses; Grass carp; Grass carp reovirus; Gill
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Grass carp, *Ctenopharyngodon idella*, a member of the family Cyprinidae, is one of the most important farmed fish species in global aquaculture, mainly in China. Production of grass carp constitutes the largest aquaculture industry in China. However, it is susceptible to several epidemics, thereinto hemorrhage disease, caused by the grass carp reovirus (GCRV), is a viral disease resulting in tremendous losses.

The innate immune system serves as the first line of protection against invading microbial pathogens through a limited number of germ line-encoded pattern recognition receptors (PRRs). PRRs recognize different pathogen-associated molecular patterns (PAMPs), then trigger innate immunity and subsequently adaptive immune response [1]. Recently, three major classes of PRRs related-recognition of viral PAMPs have been identified, including toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs) and nucleotide-oligomerization domain-like receptors (NLRs) [2].

To date, more than 13 TLR members have been found in mammals. TLRs are implicated in the detection of a vast range of pathogens including viruses, bacteria, protozoa and fungi [3]. Various viruses are perceived by intracellular TLRs (*TLR*3, *TLR*7/*TLR*8 and *TLR*9). *TLR*3 senses dsRNA, *TLR*7/ *TLR*8 mainly recognizes single-stranded RNA (ssRNA), and *TLR*9 is triggered by CpG DNA [4]. Upon viral activation, *TLR*7/*TLR*8 and *TLR*9 recruit the adaptor molecule *MyD*88 (myeloid differentiation factor 88) that initiates the induction of nuclear factor-κB (*NF-κB*) and interferon regulatory factors-3/7 (*IRF*-3/7), both of them contribute to *type* I interferon (*IFN*-I) and pro-inflammatory cytokines productions [4]. Additionally, *TLR*22 occurs exclusively in aquatic animals (teleosts and amphibians) and recognizes long-sized dsRNA [5, 6]. *TLR*3 and *TLR*22 rely on *TIR* domain-containing adaptor-inducing *IFN-β* (*TRIF*) to trigger downstream signaling cascades of *IFN*-I and pro-inflammatory cytokines [5].

RLRs consist of *RIG*-I (retinoic acid-induced protein I), *MDA5* (melanoma-differentiation-associated gene 5) and LGP2 (laboratory of genetics and physiology 2) [7]. *RIG*-I and *MDA*5 contain two N-terminal caspase recruitment domains (CARDs), a DExD/H box RNA helicase domain and a C-terminal repressor domain (RD), whereas *LGP*2 lacks the CARDs domain. The

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CARDs mediate downstream signaling cascades and induce the activation of interferon-β promoter stimulator 1 (*IPS*-1; also known as *MAVS*, *CARDIF*, or *VISA*) [8]. *IPS*-1 functions as an adaptor molecule mediating the activation of *TBK*1 (TANK-binding kinase 1) and *IKK-ε* (inhibitor of nuclear factor Iκ kinase-ε), which phosphorylate *IRF*-3/7. Then, *IRF*-3/7 translocates into the nucleus, and subsequently induces *IFN*-I and *ISGs* (IFN-stimulated genes) expressions [7].

NLRs, consisting of more than 20 members, are present in the cytosol and recognize intracellular microorganisms. *NOD*1 and *NOD*2 were significantly induced by GCRV in grass carp spleen [9]. *NOD*2 can interact with the *IPS*-1, leading to the activation of *NF-κB* and *IRF*-3, then induce the productions of cytokines and *IFN*-I in mammals [10].

In contrast to innate immunity, adaptive immunity employs antigen receptors that generate highly specific immune responses. Immunoglobulins (Igs) bind antigens with high specificity, and they are important molecules in adaptive immunity. *IgM* is evidenced to link innate immunity and adaptive immunity in mammals [11]. Additionally, teleosts produce *IgM* as a primary antibody response during the infection process.

Gill is an important tissue for processing of pathogens in environmental water [12]. Some reports about tissue expressions have indicated that gill plays important immune functions in teleosts [13,14], however, expression patterns of immune-related genes are scattered. Therefore, systematic expression profiles of classical immune-related genes can contribute to clarify immune functions of fish gill. Furthermore, better understanding immune defense mechanisms may be conducive to the development of management strategies for disease control in teleosts.

In the present study, we investigated mRNA expression profiles of twelve representative immune-related genes in grass carp gill after GCRV challenge. The results will outline immune responses to virus infection in gill.

1 Materials and methods

1.1 Fish, virus challenge, sample collection, RNA extraction and cDNA synthesis

Grass carp (15—20 g) from a fish farm were used as a source of mRNA expression analysis. Fish were acclimatized to laboratory conditions for one week in a quarantine area by maintaining in 300 L aerated aquaria at 28℃ and fed twice a day.

For viral challenge, 100 μL of GCRV (097 strain, 3.63×10^7 $TCID_{50}$/mL) per gram body weight, suspended in PBS, were injected intraperitoneally. The control animals were injected with PBS. Five individuals were sacrificed and their gills were harvested at 0h, 6h, 12h, 24h, 48h and 72h post injection.

The samples were homogenized in TRIZOL reagent (Invitrogen) and total RNAs were isolated according to the manufacturer's instruction. Total RNAs were incubated with RNase-free DNase I (Roche) to eliminate contaminated genomic DNA before being reversely transcribed into cDNA using random hexamer primers and M-MLV Reverse Transcriptase (Promega).

1.2 Virus detection

All the cDNA samples were examined for the virus by RT-PCR. The gene specific primers were designed according to the VP4 gene of GCRV 097 strain. The forward primer was S131, and reverse primer was S132 (Tab. 1).

1.3 The temporal expression profiles of TLRs, RLRs, NLRs, *MyD*88, *CiIPS*-1, *CiIFN*-I and *CiIgM* genes in grass carp gill post GCRV challenge

Quantitative real-time RT-PCR (qRT-PCR) method was established to quantify mRNA expressions of TLRs (*CiTLR*3, *CiTLR*7 and *CiTLR*22), RLRs (*CiRIG*-I, *CiMDA*5 and *CiLGP*2), NLRs (*CiNOD*1 and *CiNOD*2), adaptor molecules (*CiMyD*88 and *CiIPS*-1), *CiIFN*-I and *CiIgM* genes post GCRV injection in grass carp gill using CFX96 Multicolor Real-time PCR Detection System (Bio-Rad). The primers were listed in Tab. 1. All cDNA concentrations were adjusted to 200 ng/μL. 18S rRNA was utilized as an internal control for cDNA normalization[15]. The qRT-PCR mixture consisted of 2 μL of cDNA sample, 7.6 μL nuclease-free water, 10 μL of 2 × SYBR Green PCR master mix (TaKaRa), and 0.2 μL of each gene specific primers (10 mmol/L). The PCR cycling conditions were following: 1 cycle of 95℃ for 30s, 40 cycles of 95℃ for 5s, 60℃ for 30s, 1 cycle of 95℃ for 15s, 60℃ for 30s, 95℃ for 15s, followed by dissociation curve analysis to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the CFX96 Sequence Detection System and exported into a Microsoft Excel Sheet for subsequent data analyses. The relative expression ratios of target gene in treated group versus those in control group were calculated by $2^{-\Delta\Delta ct}$ method. Each sample was run in triplicate. The data from five independent biological replicates were subjected to one-way analysis of variance (One-way ANOVA), followed by an unpaired, two-tailed *t*-test. A value of $P<0.05$ was considered statistically significant.

Tab. 1 Primers used in this study

Gene name	Primer name	Sequence (5′-3′)	Amplicon length (bp)	GenBank accession No.
18S rRNA	18F99	ATTTCCGACACGGAGAGG	90	EU047719
	18R100	CATGGGTTTAGGATACGCTC		
*CiTLR*3	TF223	GAGAACAATCGTGACTCCCTGA	117	DQ864497
	TR224	CCAGTAGAGAACACAGCGAGGT		
*CiTLR*7	SF226	GAGCATACAGTTGAGTAAACGCAC	143	JN867639
	SR227	TCTCCAAGAATATCAGGACGATAA		
*CiTLR*22	TF483	TCGTTTGTCTGGCATTTTCTG	146	FJ547498
	TR484	CAAGGCTCGTCTTCGGTGT		
*CiMyD*88	MF213	AAAAAGGTGTAAGAGGATGGTGG	125	FJ843088
	MR214	CAGGGATTAGGCGTTTAGTGC		
CiRIG-I	RF230	ACTACACTGAACACCTGCGGAA	70	GQ478334
	RR231	GCATCTTTAGTGCGGGCG		
*CiMDA*5	MF150	CAGGAGCGACTCTTGGACTATG	103	FJ542045
	MR151	AAAGACGGTTTATTTGAATGGAAG		
*CiLGP*2	LF215	CGTCTACTCGGTGGTGGCT	124	FJ813483
	LR216	AAACTCCCTGGGACTCATACTCT		
CiIPS-1	IF217	GACCGTAAGAAGTCAGCCTCC	111	GQ483645
	IR218	CCTGAATAACTCTTGATAGCCCTC		
*CiNOD*1	NF717	CACCTACATCTTGCGATTCCC	94	FJ937972
	NR718	TTCAGGCACATTATCCAAGTCAG		
*CiNOD*2	NF719	CGGTTGAGGAGGCGGAGTC	210	FJ937973
	NR720	CCTTTACAGGACGAGGAATGGA		
CiIFN-I	IF590	AAGCAACGAGTCTTTGAGCCT	79	DQ357216
	IR591	GCGTCCTGGAAATGACCT		
CiIgM	IF725	TCTACCTCCAACTCCACCACC	185	DQ417927
	IR726	TGTTTATTGTATTTGCCACCTGAT		
*VP*4	S131	AGTTCTCAAAGCTGAGACAG	312	GQ469997
	S132	ACGTGCGATTGGAAGAGCT		

2 Results

2.1 The verification of GCRV challenge by PCR

The electrophoresis profile was exhibited at 0h, 6h, 12h, 24h, 48h and 72h post GCRV injection in both the control group and the injected group (Fig. 1). GCRV was not detected at all check points in the control group.

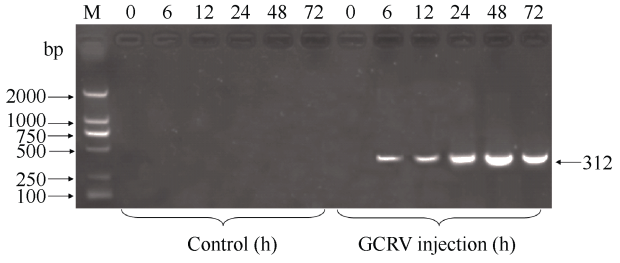

Fig. 1 Agarose gel electrophoresis profile of the PCR products was shown for virus detection at 0, 6h, 12h, 24h, 48h and 72h post GCRV injection. The fragment length was 312 bp

In contrast, viral mRNA was examined in the injected group from 24h.

2.2 Expression profiles of TLRs and *CiMyD*88 in gill

The mRNA expression profiles of TLRs (*CiTLR*3, *CiTLR*7 and *CiTLR*22) and the adaptor molecule *CiMyD*88 were measured post GCRV injection (Fig. 2A). The mRNA expressions of target genes in the control group were no significant differences among tested time points ($P>0.05$) (data not shown). The relative expressions of *CiTLR*3 were no significant differences at 6h, and largely increased from 12h (2.42 folds, $P<0.05$) to 72h (7. 98 folds, $P<0.05$). No significant change was detected in *CiTLR*7 gene expression during the course of GCRV injection at 6h ($P>0.05$). At 12h after GCRV treatment, the expression level was slightly increased (1.53 folds, $P<0.05$). As time progressed, the expression level of *CiTLR*7 mRNA was enhanced at 24h (2.09 folds, $P<0.05$). After that,

mRNA expressions were rapidly increased at 48h (8.03 folds, $P<0.05$) and 72h (12.21 folds, $P<0.05$). The expression level of *CiTLR*22 transcript was no significant difference at 6h. Then, the relative values were gradually increased from 12h (2.97 folds, $P<0.05$) to 72h (7.31 folds, $P<0.05$).

The relative expression of *CiMyD*88 was decreased at 6h (0.43 fold, $P<0.05$), then increased at 24h (1.55 folds, $P<0.05$) and reached the peak at 48h (5.12 folds, $P<0.05$). After that, the mRNA expression was still high at 72h (2.71 folds, $P<0.05$) although lower than that at 48h.

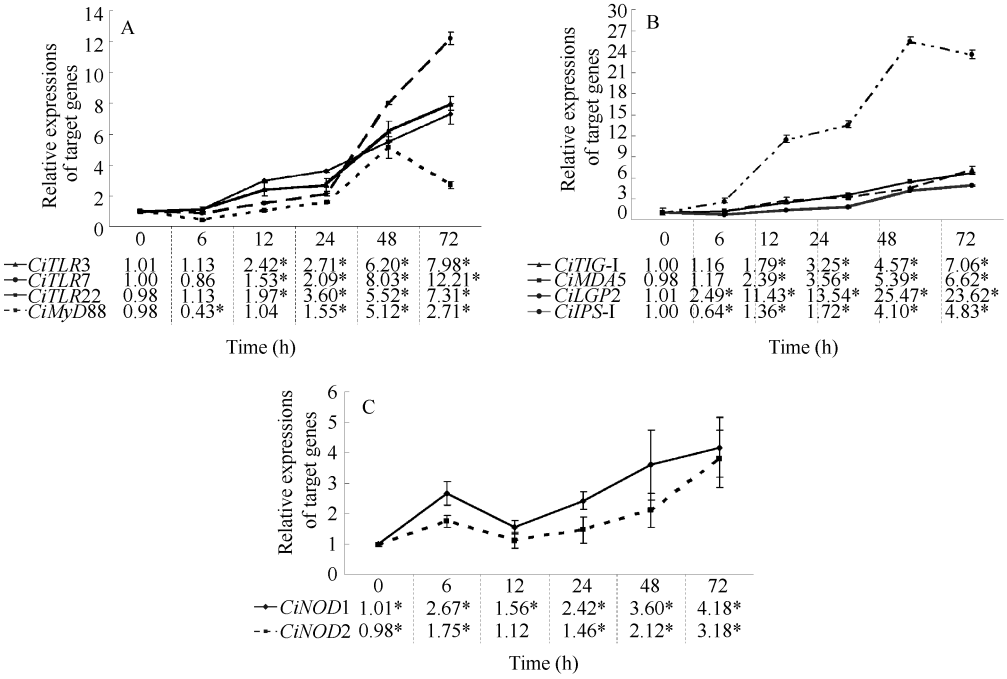

Fig. 2　mRNA expression profiles of three PRR families and their adaptors at different time points post GCRV injection in gill
A. TLRs (*CiTLR*3, *CiTLR*22 and *CiTLR*7) and *CiMyD*88; B. RLRs (*CiRIG*-I, *CiMDA*5 and *CiLGP*2) and *CiIPS*-1; C: NLRs (*CiNOD*1 and *CiNOD*2). The mRNA expressions were measured at 0, 6h, 12h, 24h, 48h and 72h. The 18S rRNA gene was used as an internal control to normalize the cDNA template. Asterisk (*) was marked significant difference ($P<0.05$) between the experimental group and the control group. Error bars indicated standard deviation

2.3　Expression patterns of RLRs and *CiIPS*-1 in gill

The mRNA expressions of RLRs (*CiRIG*-I, *CiMDA*5 and *CiLGP*2) and *CiIPS*-1 were examined in gill post viral challenge (Fig. 2B). The expression levels of *CiRIG*-I were gradually increased from 12h (2.79 folds, $P<0.05$) to 72h (7.06 folds, $P<0.05$). Similarly, the relative quantities of *CiMDA*5 were increased between 12h (2.39 folds, $P<0.05$) and 72h (6.62 folds, $P<0.05$). The relative values of *CiLGP*2 were up-regulated at 6h (2.49 folds, $P<0.05$), and sharply increased from 12h (11.43 folds, $P<0.05$) to 72h (23.62 folds, $P<0.05$).

Interestingly, the relative value of *CiIPS*-1 was down-regulated to 0.64-fold ($P<0.05$) at 6h. After that, the expression levels were slightly increased at 12h (1.36 folds, $P<0.05$) and 24h (1.72 folds, $P<0.05$), then rapidly enhanced at 48h (4.10 folds, $P<0.05$) and 72h (4.83 folds, $P<0.05$).

2.4　Expression tendencies of NLRs in gill

The time-dependent expression patterns of *CiNOD*1 were shown in Fig. 2C. After GCRV challenge, the mRNA expression of *CiNOD*1 began to increase at 6h (2.67 folds, $P<0.05$). The relative expressions were slightly enhanced at 12h (1.56 folds, $P<0.05$). After that, the relative levels of *CiNOD*1 were up-regulated at 24h (2.42 folds, $P<0.05$), 48h (3.60 folds, $P<0.05$) and 72h (4.18 folds, $P<0.05$). Similarly, mRNA expressions of *CiNOD*2 were shown in Fig. 2C. The relative value was slightly increased at 6h (1.75 folds, $P<0.05$). Afterwards, the mRNA expression of *CiNOD*2 was decreased to the control level at 12h. Then, the relative quantities were gradually enhanced from 24h (1.46 folds, $P<0.05$) to 72h (3.81 folds, $P<0.05$).

2.5　Expression patterns of *CiIFN*-I in gill

The time-dependent expression patterns of *CiIFN*-I were examined (Fig. 3A). The expression levels of *CiIFN*-I were slightly increased at 6h (1.44 folds, $P<0.05$), then rapidly enhanced from 12h (2.81 folds, $P<0.05$) to 72h (8.30 folds, $P<0.05$).

2.6　Expression profiles of *CiIgM* in gill

The mRNA expression profiles of *CiIgM* were tested (Fig. 3B). There were no significant differences of *CiIgM* expressions from 0h to 48h. Then, the mRNA expression of *CiIgM* was enhanced at 72h (2.85 folds, $P<0.05$).

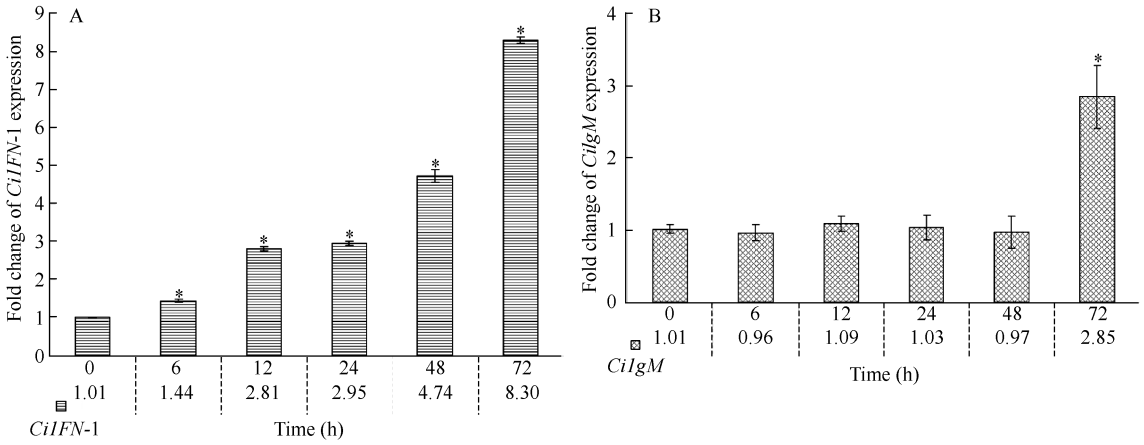

Fig. 3 The mRNA expression patterns of *CiIFN*-I and *CiIgM* at different time points after GCRV injection in gill. A: *CiIFN*-I; B: *CiIgM*. Other captions are same as those in Fig. 2

3 Discussion

Fish gill contacts with external water environment, and it is a vital organ to defense the invasion of different pathogens [12]. GCRV is endogenic virus, and intraperitoneal injection is superior to immersion or co-habitation challenge for virus invasion [16]. According to the profile of agarose electrophoresis (Fig. 1), it showed that GCRV successfully infected grass carp.

mRNA expression of TLR3 is significantly up-regulated by GCRV challenge in spleen at day 1 in grass carp [17] and in gill at 12h in rare minnow [18]. The relative expressions of *CiTLR*3 in gill were increased from 12h to 72h post GCRV injection (Fig. 2A) and the data implied that *TLR*3 played a role in inducing the innate immune response against virus in grass carp gill. mRNA expressions of *CiTLR*22 are significantly increased at 6h, then rapidly drop to control levels from 12h to 48h post GCRV injection in spleen [6]. In the present study, the expression of *CiTLR*22 was increased at 12h post GCRV challenge in gill (Fig. 2A). The relative value in gill was lower than that in spleen at 6h, however, the mRNA expression showed a more persistent up-regulation in gill. Taken together, the results indicated that *CiTLR*22 displayed different expression profiles in different tissues post GCRV challenge. The tendencies of *CiTLR*3 and *CiTLR*22 were nearly the same at examined time points in gill (Fig. 2A). Therefore, *TLR*22 might serve as a surveillance coupled with *TLR*3 for dsRNA virus recognition in grass carp. *TLR*7 recognizes viral ssRNA inducing the production of *IFN*-I and inflammatory cytokines in mammals [4]. Additionally, *TLR*7 expression level in rainbow trout anterior kidney leukocytes is not affected by poly(I:C) treatment [19]. Interestingly, the expression of *TLR*7 was significantly up-regulated after GCRV injection in grass carp gill (Fig. 2A). The previous study has implied that *TLR*7 is involved in response to dsRNA virus in grass carp [20, 21]. The results further evidenced that *TLR*7 might participate in the recognition of dsRNA virus in grass carp, which provided a new sight for systematic research of immune signaling pathways in teleosts. The mRNA expressions of *CiMyD*88 were decreased at 6h post GCRV challenge, and gradually increased from 12h to 72h (Fig. 2A). The down-regulation of *MyD*88 at the early stage might be explained that the expression of *MyD*88 was suppressed by GCRV injection. Therefore, *MyD*88 was an important regulator in response to GCRV challenge in grass carp gill.

Generally, *RIG*-I and *MDA*5 discriminate different PAMPs against RNA viruses in mammals [7]. mRNA expressions of channel catfish *RIG*-I and *MDA*5 are increased in channel catfish ovarian cells post channel catfish virus (CCV) challenge [22]. In the present research, both *RIG*-I and *MDA*5 were also up-regulated post GCRV injection in grass carp gill (Fig. 2B). Similarly, common carp *RIG*-I is up-regulated in spleen, head kidney and intestine tissues after SVCV (spring viraemia of carp virus) infection [23]. It has been reported that the expression of *RIG*-I is dramatically enhanced in EPC cell post VHSV (viral hemorrhagic septicemia virus) infection [24]. Collectively, *CiRIG*-I and *CiMDA*5 were critical for the activation of antiviral innate immunity. Mammalian *LGP*2 acts as a negative regulator of *IFN*-I production and antiviral signaling [25], but Japanese flounder *LGP*2 exhibits positive function in response to both ssRNA and dsRNA viruses [26]. In addition, *LGP*2 shows a positive role post VHSV infection in rainbow trout [27]. In the present study, *LGP*2 exhibited a powerful up-regulation, especially at 48h and 72h in grass carp gill (Fig. 2B). The expression patterns of *IPS*-1 were discrepant after virus challenge in teleosts. *IPS*-1 is down-regulated post poly(I:C) stimulation or ISKNV (infectious spleen and kidney necrosis virus) challenge in spotted

green pufferfish spleen [28]. Whereas, common carp *IPS*-1 are up-regulated in spleen, head kidney and intestine tissues post SVCV challenge [23]. In accord with EPC cells and common carp, the expression of *IPS*-1 was mainly enhanced from 12h to 72h post GCRV challenge in grass carp (Fig. 2B). Interestingly, the relative value of *IPS*-1 was slightly reduced at 6h (Fig. 2B), which was the similar to the expression of *MyD*88 in grass carp gill (Fig. 2A). The data further implied that *IPS*-1 played a vital role in antiviral immune response against GCRV in grass carp gill.

NLRs and RLRs are two major classes of cytoplasmic PRRs in innate immune system. In general, RLRs mediate antiviral defense, whereas NLRs primarily elicit antibacterial function. However, the mRNA expressions of *NOD*1 and *NOD*2 are significantly increased upon GCRV infection in grass carp spleen [9]. In the present study, GCRV also could induce the expression of *NOD*1 and *NOD*2 in grass carp gill (Fig. 2C). Furthermore, the relative quantities of *CiNOD*1 were higher than those of *CiNOD*2 at corresponding time points (Fig. 2C), which was opposite to that in spleen [9]. Collectively, *NOD*1 and *NOD*2 had a role in immune protection in response to viral invasion in grass carp.

During virus infection, multiple signaling cascades are activated, leading to the production of *IFN*-I to inhibit viral replication. To data, a variety of *IFN* genes have been identified in teleosts [29, 30]. The expression of *CiIFN*-I mRNA was gradually increased by GCRV challenge in gill (Fig. 3A), which provided the evidence that *IFN*-I played a critical role in innate immunity against viral invasion in grass carp gill.

Teleosts have both innate and adaptive immune systems to combat viral infection. *IgM* is mainly detected in gill, head kidney, intestine, liver, trunk kidney and spleen by semi-qRT-PCR in grass carp [31]. The expression levels of *CiIgM* were no differences at examined time points except at 72h (Fig. 3B), which indicated that the adaptive immunity was initiated at three days after GCRV injection in grass carp gill.

In summary, mRNA expression patterns of 12 immune-related genes were checked in grass carp gill post GCRV challenge. The results indicated that gill is an important immune organ to resist virus in grass carp. The findings contribute to comprehensive clarification of antiviral immunity in teleosts and lay a foundation for development of management strategies in disease control.

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草鱼鳃介导草鱼呼肠孤病毒免疫应答

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摘要: 采用草鱼呼肠孤病毒腹腔注射草鱼, 通过定量 RT-PCR 检测了 12 个抗病毒免疫相关基因在鳃中不同时间点的表达模式, 以了解鳃对内源性病毒的免疫应答。模式识别受体基因 *CiTLR*3、*CiTLR*7、*CiTLR*22、*CiRIG*-I、*CiMDA*5、*CiLGP*2、*CiNOD*1 和 *CiNOD*2, 以及干扰素基因 *CiIFN*-I 的表达在注射病毒后 12h、24h、48h 及 72h 基本都上调。IgM 基因的表达仅在 72h 上调。接头分子 *CiMyD*88 和 *CiIPS*-1 基因的表达在早期下调(6h), 然后逐渐上升。为了证实病毒感染的可靠性, 通过 RT-PCR 检测了病毒 *VP*4 基因。结果表明草鱼鳃在抗病毒免疫方面发挥着重要作用。

关键词: 免疫应答; 草鱼; 草鱼呼肠孤病毒; 鳃