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Molecular characterization of Cyprinid herpesvirus 3 encoded viral interleukin10

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ABSTRACT

Cyprinid herpesvirus 3 (CyHV-3), a virus that encodes an interleukin10 (IL-10) homologue, causes severe economic losses to the common carp and koi culture industry. The present study was devoted to this IL-10 homologue. Recombinant viral IL-10 (vIL-10) protein encoded by CyHV-3 ORF134 gene using prokaryotic expression system was obtained successfully. Bioinformatics analysis revealed that the amino acid sequence of CyHV-3 vIL-10 has low homology with other host IL-10 or viruses encoded IL-10s. However, their tertiary structure is quite similar, suggesting conservative biological functions between IL-10s and vIL-10s. The biological activity of CyHV-3 vIL-10 was detected by using CCK-8 kit and real time quantitative PCR. The results showed that CyHV-3 vIL-10 down regulate epithelioma papulosum cyprini (EPC) cellular activity at 72 h. Moreover, CyHV-3 vIL-10 inhibits the LPS-induced expression of proinflammatory genes, similar to common carp IL-10. Altogether, the results of this study demonstrate that a clear biological activity of CyHV-3 vIL-10 on its host cells and indicates CyHV-3 vIL-10 may play an important role in viral immune evasion.

1. Introduction

Interleukin 10 (IL-10) is one of the most important anti-inflammatory cytokines for host immunity, with both immunostimulative and immunosuppressive properties [1]. IL-10 regulates and suppresses the expression of proinflammatory cytokines during the recovery phases of infections and consequently reduces the damage caused by inflammatory cytokines [2]. During the infection process, a variety of viruses avoid host immune clearance by up-regulating the expression of IL-10 [3–6]. However, many viruses have developed immune evasion mechanisms in order to establish latent and persistent infection [7]. Among these mechanisms, large DNA viruses encode or express protein homologs of cytokines and chemokines [7–9]. To this end, virally encoded IL-10 homologs, also called viral IL-10 (vIL-10), have been reported in members of the family *Herpesviridae*, *Alloherpesviridae* and *Poxviridae* [7]. Almost all viruses which encode IL-10 homologs infected mammalian hosts, with only two viruses exceptions that both are present in fish. One is anguillid herpesvirus 1 (AngHV-1) which infects European eel (*Anguilla anguilla*), and other one is cyprinid herpesvirus 3 (CyHV-3) which infects common carp (*Cyprinus carpio*) [10]. Both of them belong to *Alloherpesviridae*. Thus, CyHV-3 is one of only few nonmammalian viruses known to express an IL-10 homologue.

CyHV-3, also known as koi herpesvirus (KHV), a pathogen of koi herpesvirus disease (KHVD), causes high morbidity and mortality in koi and common carp (*Cyprinus carpio*). This disease has caused severe economic losses in both koi and common carp aquaculture industries globally. Aoki et al. sequenced the whole genome of CyHV-3 and predicted the genes that can participate in viral immune evasion [11]. CyHV-3 ORF 134 encoded IL-10 homologue has been predicted [11]. Previous studies showed that expression of CyHV-3 vIL-10 gene was high in gill, kidney and spleen during the acute or reactivation phases of CyHV-3 infection [12]. But during the persistent phase of CyHV-3 infection, these were lower levels of vIL-10 expression in same tissues of infected carp [12]. Interestingly, comparison of a strain deleted for ORF134 and a derived revertant strain demonstrated that ORF134 is not essential neither for CyHV-3 replication *in vitro* nor for virulence in

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vivo in common carp [13]. Recent studies confirmed that CyHV-3 vIL-10 acts through a conserved signaling pathway, involving Stat3 phosphorylation and induction of *socs3* expression similar to IL-10 [1,14].

In the current study, recombinant CyHV-3 vIL-10 was used to analyze it for comprehensive bioinformation and to assess its activity on EPC cells or macrophages of common carp. CyHV-3 vIL-10 alone stimulation hardly causes expression of immune-related genes in macrophages. However, it affected the activity of EPC cells significantly. Furthermore, CyHV-3 vIL-10 inhibited the LPS-induced immune-related genes expression. This study reveals the distinct function of CyHV-3 vIL-10.

2. Materials and methods

2.1. Bioinformatics analysis

The bioinformatics analysis of CyHV-3 vIL-10 (ABG42961) by following tools. The Protparam tool (https://www.expasy.org/tools) was used for physicochemical properties analysis. Multiple sequences alignments and phylogenetic analysis by the Clustal X 2.0 and Mega 6.0. Signal peptides and transmembrane helices were predicted respectively using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The KinasePhos (http://kinasephos.mbc.nctu.edu.tw/) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) were used for phosphorylation and glycosylation prediction respectively. Secondary structure and tertiary structure of protein were predicted respectively using the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/), SWISS-MODEL (https://www.swissmodel.expasy.org/) and PyMOL software.

2.2. Cells and virus

Common carp brain cells (CCB) were cultured in dulbecco's modified eagle medium (DMEM) (HyClone) maintained 10% fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin (Solarbio), and 100 μg/mL streptomycin (Solarbio). The CyHV-3 used in this study was isolated from common carp during a disease outbreak in China, Chengdu, in 2015 [15]. CCB cells were infected with CyHV-3 and cultured at 25 °C in a humid atmosphere containing 5% CO_2.

2.3. Production of recombinant CyHV-3 vIL-10

Total RNA was isolated from infected cell cultures using the RNAiso Plus (Takara) according to the product manual. cDNA was synthetized from RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) according to the product manual. The mature form of CyHV-3 vIL-10 (ABG42961) without signal peptide was amplified from infected cell cultures cDNA using specific primers including additional *BamH* I and *Hind* III sites (Table 1) and delivered into pMD19-T vector (Takara). The gene was subsequently cloned between the *BamH* I and *Hind* III restriction sites in-frame with the hexahistidine tag of pET32a expression vector and transformed into BL21 (DE3) competent *E. coli* (TIANGEN). Bacteria containing the CyHV-3 vIL-10 plasmid were plated on lysogeny broth (LB) agar plates (supplemented with 100 μg/mL ampicillin) and incubated overnight at 37 °C. Positive clones were identified by colony PCR using vector-specific primers, and the products were sequenced by Sangon Biotech. Selected positive clones were used to inoculate 500 mL LB medium (supplemented with 100 μg/mL ampicillin) and incubated on shaker at 150 r/min at 37 °C until an OD_{600} of 0.6–0.8 was reached. Protein expression was induced with 0.4 mM isopropyl β-D thiogalactoside (IPTG) and continued culturing for 4 h at 37 °C. After incubation, bacteria were pelleted by centrifugation at 8000 r/min for 10 min, resuspended in 25 mL binding buffer I containing 50 mM Tris-HCl, 250 mM NaCl, 0.05% Triton-100 (v/v). Bacteria were lysed by ultrasound after three times of freeze-thawing. Inclusion bodies were pelleted by centrifugation at 8000 r/min for 30 min, washed with 25 mL binding buffer II containing 50 mM Tris-HCl, 250 mM NaCl, 2 M urea, and 0.05% Triton-100 (v/v) and solubilized in 25 mL binding buffer III containing 50 mM Tris-HCl, 250 mM NaCl, 8 M urea, and 0.05% Triton-100 (v/v). After purification through a 0.22 μm filter, hexahistidine-tagged proteins were purified by Bio-Scale™ Mini Nuvia™ IMAC Ni-Charged (BIO-RAD). Briefly, the solubilized inclusion bodies were adsorbed in Ni column, and washed with washing buffer containing 50 mM Tris-HCl, 250 mM NaCl, 8 M urea, 20 mM imidazole and 0.05% Triton-100 (v/v). Proteins were eluted by elution buffer containing 50 mM Tris-HCl, 250 mM NaCl, 8 M urea, 200 mM imidazole and 0.05% Triton-100 (v/v). The purified proteins were dialyzed against binding buffer containing urea with gradient concentration, centrifuged at 12,000 rpm to remove any precipitate, filtered sterilized, mixed with 20% (v/v) glycerol, and stored at −80 °C. The purified proteins were assessed by SDS-PAGE and the concentration was assessed by NanoDrop 1000 spectrometer (Thermo Scientific).

Table 1
Primers used for cloning and real time quantitative PCR.

Primers	Sequence (5′-3′)	GenBank Number
vIL-10-F	GGATCCCCAGCTACTACGCCGAA	ABG42961
vIL-10-R	AAGCTTGTTTGCGCTTGGTTTTCAT	
β-actin-F	GACCTGTATGCCAACACTGTAT	M24113
β-actin-R	TCCTGCTTGCTAATCCACATC	
40 s-F	CCGTGGGTGACATCGTTACA	AB012087
40 s-R	TCAGGACATTGAACCTCACTGTCT	
IL-1β-F	GCTCGGCTTCATCTTGGAGAATGT	AJ245635
IL-1β-R	GCAAGGTGAGGCTGGTCTTATTGT	
INOS-F	AACAGGTCTGAAAGGGAATCCA	AJ242906
INOS-R	CATTATCTCTCATGTCCAGAGTCTCTTCT	
TNF-α-F	AGGTGATGGTGTCGAGGAGGAAG	AJ311800
TNF-α-R	AGACTTGTTGAGCGTGAAGCAGAC	
IL-6-F	TGAAGACAGTGATGGAGCAGCAGA	KC858890
IL-6-R	CCTCACAGCAATGTGGCGAACA	
IFN-γ1-F	AAGGAACCGTCGGAGCCAGAC	AM261214
IFN-γ1-R	ATCAAGCAGCAGCGACTGACAAG	
IL-12-F	TGCTTCTCTGTCTCTGTGATGGA	AJ580354
IL-12-R	CACAGCTGCAGTCGTTCTTGA	
IL-10-F	AGTCCTTATGGCTGTCACGTCATG	AB110780
IL-10-R	TTGAGTGCAAGTGGTCCTTCTGG	
IL-8-F	GCTGCACTGCTGTCCACAA	AB470924
IL-8-R	TAGCAGGAATTGCTGGCTCTG	
MHC I-F	GACTGCTGCCAATCCTCAAGC	AB018581
MHC I-R	TCTCTCCAGAGTGTCCTTGCCATA	
CXCa-F	CTGGGATTCCTGACCATTGGT	AJ421443
CXCa-R	GTTGGCTCTCTGTTTCAATGCA	

2.4. Western blot analysis

The purified proteins were mixed with 5 × SDS-PAGE Loading Buffer (Solarbio) and resolved on 12% SDS-PAGE gel. Then the prepared protein sample was transfer to PVDF membrane (Sangon Biotech) and blocked for 1 h at 60 rpm at 37 °C with 3% BSA (Solarbio) in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20) then that an overnight incubation was done at 4 °C with a 1:1000 dilution of anti-6 × His rabbit polyclonal antibody (Sangon Biotech) in 0.5% BSA TBST. A 1:8000 dilution of HRP-conjugated goat anti-rabbit Abs (Sangon Biotech) in 0.5% BSA TBST was used as secondary Abs and incubated at 60 rpm at 37 °C at least for 1 h. Final results were visualized by Enhanced HRP-DAB Chromogenic Substrate Kit (TIANGEN).

2.5. Detection of cellular activity by CCK-8 kit

The effect of CyHV-3 vIL-10 on cellular activity of epithelioma papulosum cyprini (EPC) cells was detected using CCK-8 kit (Solarbio)

according to the manufacturer's instructions. Briefly, EPC cells (6×10^4 cells/well) were pre-cultured in 96-well plate (Corning) for 24 h. EPC cells were stimulated with rvIL-10 (1 μg/mL) or tag protein (rpET32a) (1 μg/mL) for 24, 48, 72 and 96 h. After incubating 10 μL of CCK-8 per well for one and a half hours at each time point, OD_{450} was measured by Thermo Scientific Varioskan Flash (Thermo Scientific).

2.6. Fish

Common carp were obtained from an aquatic farm in Guangyuan, Sichuan, China. Common carp were kept in 60-L tanks at 24 °C. Microbiological, clinical and parasitical examination of the fish demonstrated that these fish were fully healthy.

2.7. Cell isolation and culture

Common carp were slaughtered for organ collection. Head kidney-derived macrophages were isolated as described [16]. Briefly, head kidney cell suspensions were filtered through 100 mesh cell filter that were layered on 51% (1.071 g/cm^3) Percoll (Solarbio) and centrifuged at 400 × *g* for 30 min at 4 °C. Cells at the medium/Percoll interface were removed and washed twice. After cells counting by 0.4% trypan blue (Solarbio) stating, cells (2.5×10^6 cells/well) were cultured in 6-well plate (Corning).

2.8. Gene expression analysis by real-time quantitative PCR

To measure changes in gene expression, 2.5×10^6 cells were stimulated with rvIL-10 (0.1 μg/mL or 1 μg/mL or 10 μg/mL) and/or LPS (20 μg/mL) for 3 h. Tag protein (rpET32a) (1 μg/mL) was used as a blank control. Total RNA was isolated using RNAiso Plus (Takara) according to the manufacturer's instructions, and stored at −80 °C. cDNA was synthesized for real-time quantitative analysis using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions, and stored at −20 °C. RT-qPCR analysis was performed with a StepOnePlus (Applied Biosystems) using TB Green™ Premix Ex Taq™ II (Takara). The primers used for RT-qPCR are listed in Table 1. Fluorescence data from RT-qPCR experiments were analyzed using the StepOnePlus real-time PCR system and exported to Microsoft Excel sheets. The threshold cycle (Ct) was determined using the Auto method for all runs. The expression of analyzed genes was calculated using the $2^{-\Delta\Delta Ct}$ method [17]. The 40s and β-actin were used as reference genes.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 17.0. One-way ANOVA was used for multiple comparisons, followed by the Tukey test. In all cases, asterisks (*) indicate significant differences from two groups comparing each other. * indicate $P < 0.05$, ** indicate $P < 0.01$, ***indicate $P < 0.001$.

3. Results

3.1. Analysis of protein sequence of CyHV-3 vIL-10

CyHV-3 vIL-10 has a polypeptide consisting 179 amino acids. Its molecular formula is $C_{890}H_{1409}N_{237}O_{268}S_{12}$ and its relative molecular mass is 20.1021 kDa predictively. According to the analysis of its amino acid composition, the higher amino acids in the protein were Ala (8.4%) and Leu (8.4%), and the lower content amino acid was Trp (0.6%). vIL-10 signal peptide was predicted that N 1–17 (MFLAVLLTATIFFEARG) was a possible signal peptide sequence, and its signal peptide cleavage site was located at the 17th position G (Gly) and the 18th place A (Ala) between amino acid sequences (Supplementary Fig. 1A). In addition, the transmembrane region of vIL-10 prediction

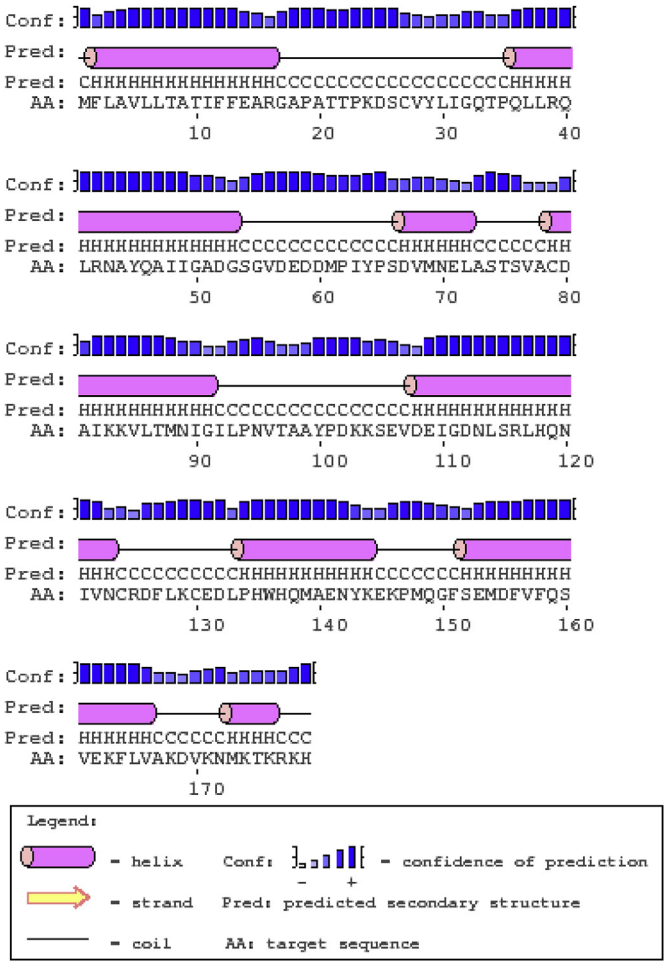

Fig. 1. Protein secondary structure prediction.

showed that vIL-10 does not contain a transmembrane region (Supplementary Fig. 1B). The vIL-10 amino acid sequence contains one potential threonine (Thr) phosphorylation site and two potential N-glycosylation sites. The phosphorylation of Thr at the 22nd position (Supplementary Fig. 1C). While at the 0.5 threshold, the 95th and 113th position of asparagine (Asn) may be glycosylated (Supplementary Fig. 1D).

The secondary structure of CyHV-3 vIL-10 was predicted that the vIL-10 consists of alpha-helix and random coil, accounting for 55.3% and 44.7%, respectively (Fig. 1). This sequence does not contain an extended strand and a beta turn (Fig. 1). Tertiary structure of common carp IL-10 and viruses encoded IL-10 were predicted that CyHV-3 vIL-10 not only has a very high similarity to the tertiary structure of common carp IL-10 but also other viruses (EBV, EHV-2, GPV, ORFV, OvHV-2 and AngHV-1) encoded IL-10 suggesting their function is conservative (Fig. 2).

3.2. Multiple sequences alignment

The amino acid sequence of IL-10 from human (NP_000563) and common carp (AFV36669), and vIL-10 from CyHV-3 (ABG42961) were aligned. There is low similar sequence between them. The four cysteines, conserved across all IL-10 genes that pair to make up the two disulfides bridges, are present in CyHV-3 vIL-10 at positions 27, 79, 124 and 130 respectively (Fig. 3). Comparison of the structure of human IL-10, CyHV-3 vIL-10 possesses a characteristic a-helical fold consisting of six helices (A–F) [18]. There are six conserved residues of CyHV-3 vIL-10 that participate in the IL-10/sIL-10R1 interface similar to human IL-

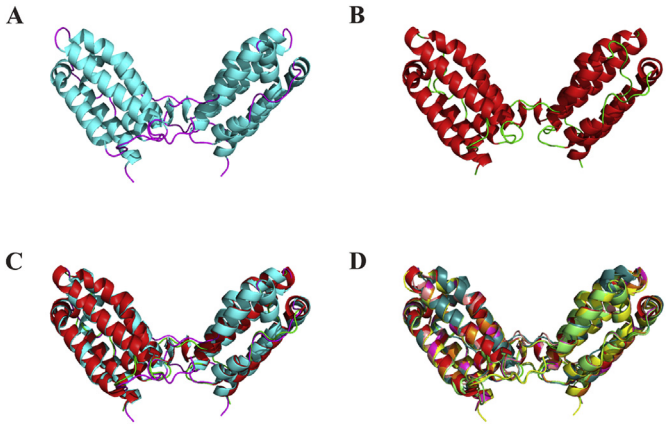

Fig. 2. Protein tertiary structure predictions. (A) Tertiary structure of common carp IL-10, helix and loop are colored blue and pink respectively. (B) Tertiary structure of CyHV-3 vIL-10, helix and loop are colored red and green respectively. (C) Tertiary structure of common carp IL-10 (blue) and CyHV-3 vIL-10 (red). (D) Tertiary structure of CyHV-3 vIL-10 (red), EBV vIL-10 (pink), EHV-2 vIL-10 (yellow), GPV vIL-10 (flesh), ORFV vIL-10 (orange), OvHV-2 vIL-10 (light green) and AngHV-1 vIL-10 (dark green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

10 [19]. Three of the six residues are found in site I a and three are found in site I b. These six amino acids form a conserved sIL-10R1 binding site in the center of site I [19]. Like IL-10, CyHV-3 vIL-10 also contains signal peptides of similar length (Fig. 3). In addition, Amino acid sequence comparisons were made between CyHV-3 vIL-10 and other vIL-10s (Fig. 4). Although they are all virus encode IL-10 homologs, their sequence similarity is not high. The four cysteine residues that pair to make up the two disulfide bridges are not only conserved in all IL-10 genes but also in all vIL-10 genes (Fig. 4).

3.3. Phylogenetic tree construction

Phylogenetic analysis was performed using vIL-10 gene sequence from 20 viruses and CyHV-3 (ABG42961). The result shows that CyHV-3 vIL-10 has a long relationship with other vIL-10s (Fig. 5). The virus from same genus has similar relationship, except CyHV-3 and AngHV-1. Although they are the only two nonmammalian viruses that encode IL-10 homologs both from the family *Alloherpesviridae* and the genus *Cyprinivirus*, they are clearly distinct from each other. Compared with *cytomegalovirus*, CyHV-3 vIL-10 is slightly closer to *lymphocryptovirus* even though they all are herpesvirus (Fig. 5). Furthermore, there is another phylogenetic analysis among fish IL-10 genes, human IL-10 gene, human IL-20 gene and CyHV-3 vIL-10 gene. The result shows that in line with the low sequence identity between CyHV-3 vIL-10 gene and its host (common carp) IL-10 (Fig. 6). The IL-10 family of cytokines can be categorized into three subgroups (IL-10 itself, the IL-20 subfamily and the type III IFN group) based primarily on biological functions [20]. Through phylogenetic tree analysis, CyHV-3 vIL-10 ends up at the basis of the group of IL-10, clearly distinct from human IL-20 (Fig. 6).

3.4. The effects of EPC cellular activity on CyHV-3 vIL-10

Purified recombinant CyHV-3 vIL-10 protein obtained from prokaryotic expression system (Supplementary Figs. 2–4). To investigate whether CyHV-3 vIL-10 exerts the cellular activity on EPC cells, EPC cells were stimulated with rvIL-10 alone and OD_{450} was measured after incubation with CCK-8 (Fig. 7). There was no significant difference in cellular activity between each group after 24 h and 48 h. But at 48 h, although not statistically significant, cellular activity was upregulated which stimulated with vIL-10. At 72 h and 96 h, cellular activity was significantly downregulated in vIL-10 stimulated group, especially at 72 h. Overall, these data suggest that CyHV-3 vIL-10 exerts downregulated activities in the late stage on EPC cells.

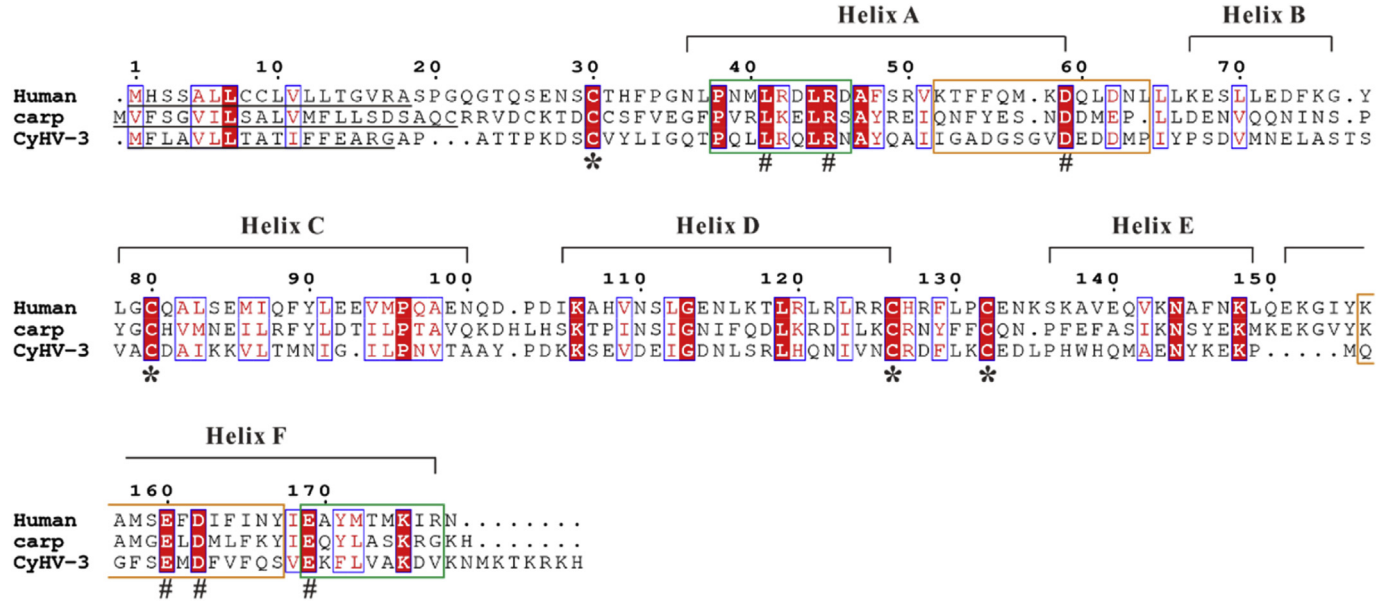

Fig. 3. Amino acid sequence alignment of IL-10 from human (NP_000563) and common carp (AFV36669), and vIL-10 from CyHV-3 (ABG42961). The position of the helices A to F was indicated by continuous lines as determined in human IL-10 [18]. Boxes indicate the human IL-10R1 binding sites: orange lines (I a) and green lines (I b) [19]. Six conserved residues that participate in the IL-10/sIL-10R1 interface are indicated in hashtag (#) [19]. The four conserved cysteine residues that pair to make up the two disulfide bridges are indicated in asterisk (*). The signal peptides of each sequence are indicated by continuous lines below the numbering. Consensus sequence is shown by white font and red background. Greater than or equal to 70% homologous sequences are shown by red font. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

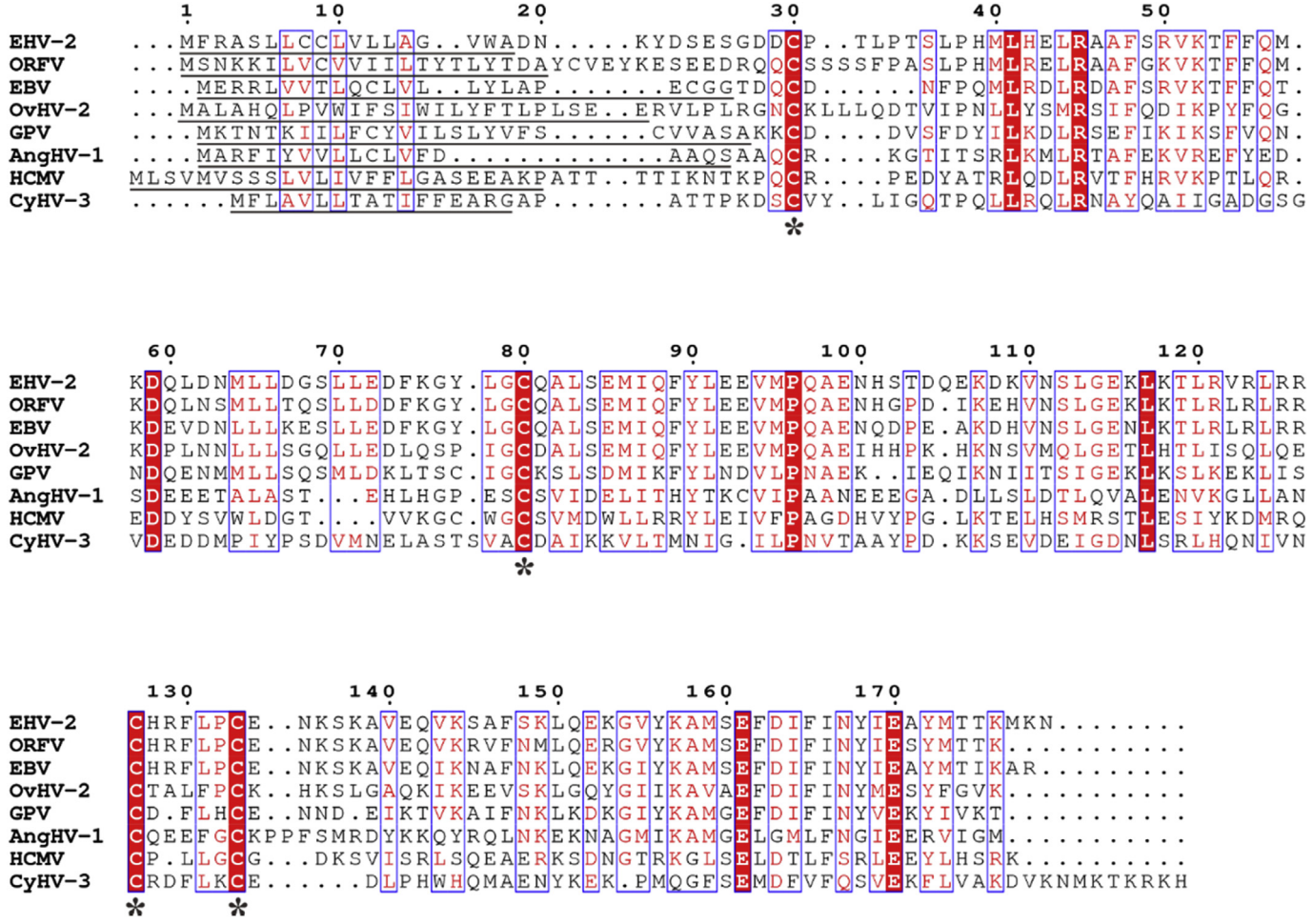

Fig. 4. Amino acid sequence alignment of vIL-10 from EHV-2 (AAC13857), ORFV (AAR98352), EBV (CAD53385), OvHV-2 (AAX58040), GPV (YP_001293197), AngHV-1 (AFK25321), HCMV (AAR31656) and CyHV-3 (ABG42961). The four conserved cysteine residues that paired to make up the two disulfide bridges are indicated in asterisk (*). The signal peptides of each sequence are indicated by continuous lines below the numbering. Consensus sequence is shown by white font and red background. Greater than or equal to 70% homologous sequences are shown by red font. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. The effects of the expression of immune-related genes on CyHV-3 vIL-10 stimulation

To assess the direct modulatory effects of CyHV-3 vIL-10 on immune-related genes expression, head kidney-derived macrophages were stimulated for 3 h with just increased concentrations of vIL-10, and the expression of several proinflammatory genes, chemokine, as well as gene involved in Ag presentation (Table 1) was measured. Gene expression of IL-1 β, TNF-α, IFN-γ1, CXCa, INOS, IL-12, IL-8 were significantly upregulated by LPS stimulation. However, when stimulated with increased concentrations of vIL-10, their gene expression was not different, comparing with the control group (Fig. 8). No significant upregulation of others immune-related genes expression (IL-10, MHC I and IL-6) were observed in macrophages stimulated with LPS or vIL-10, except one group. Significant increase in IL-10 expression after stimulation with high concentrations of vIL-10 was observed. Although not statistically significant, vIL-10 could inhibit MHC I gene expression.

3.6. The effects of the LPS-induced expression of immune-related genes on CyHV-3 vIL-10 stimulation

To assess the modulatory effects of CyHV-3 vIL-10 on LPS-induced immune-related genes expression, head kidney-derived macrophages were stimulated for 3 h with increased concentrations of vIL-10 in combination with LPS, and the expression of several proinflammatory genes, chemokine, as well as gene involved in Ag presentation (Table 1) was measured. CyHV-3 vIL-10 strongly inhibited the LPS-induced gene expression of TNF-α and INOS in macrophages, especially medium concentration of vIL-10 (Fig. 9). Although not statistically significant, downregulation of IL-1β was found in macrophages stimulated with LPS in the presence of low or medium concentration of vIL-10, while stimulation of high concentrated vIL-10 was not affect IL-1β expression (Fig. 9). What is interesting is that low concentrated vIL-10 inhibited IFN-γ1 expression while high concentrated vIL-10 significantly upregulation its expression unexpectedly. The gene expression of CXCa and IL-12 had a downward trend after stimulation of vIL-10. The inhibitory effect was not significant on IL-10, MHC I, IL-8 and IL-6 in macrophages, possibly also as a result of the low induction of these cytokines' gene expression by LPS. However, the gene expression of MHC I showed a downward trend.

4. Discussion

Inhibition of cellular immunity is the main function of IL-10. IL-10 can not only inhibit, directly or indirectly, the maturation of T lymphocytes, but also promote the proliferation and maturation of B lymphocytes. Under the long-term selection pressure, some viruses in order to escape or inhibit the host immune attack encoded an IL-10 homologue which can imitate the biological function of IL-10. According to the summary, vIL-10 orthologues have been reported 21 members from

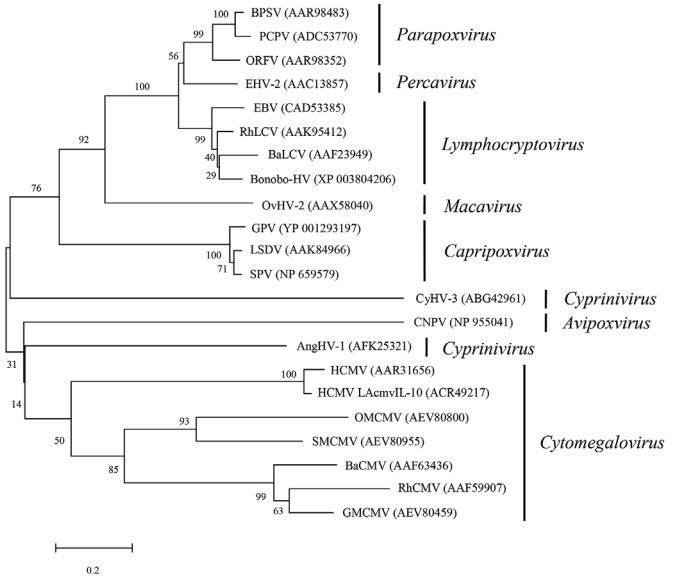

Fig. 5. Phylogenetic tree depicting the relationship between CyHV-3 vIL-10 gene and other vIL-10 genes inferred by the neighbor-joining method using the Mega 6.0. Reliability of the branching is indicated at the nodes as the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates).

the family *Herpesviridae*, *Alloherpesviridae* and *Poxviridae* [7]. These reported viruses encoding vIL-10 mostly infect higher vertebrates [7]. At present two viruses encode for vIL-10 among those viruses that infect lower vertebrates [10]. It is suggested that lower vertebrate viruses may also have immune evasion mechanisms similar to other viruses.

The ORF134 coding sequence of CyHV-3 Chinese strain was obtained by RT-PCR in the present study. The sequencing showed that the sequence was identical to the ORF134 sequence encoded by other CyHV-3 isolates. Previously viral structural proteomic of CyHV-3 studies suggested that vIL-10 was a non-structural gene [21,22]. The findings of this study further confirm that CyHV-3 vIL-10 is a functional non-structural gene.

Functional prediction of amino acid sequence from CyHV-3 vIL-10 indicated that it is a secreted protein and a member of the IL-10 superfamily. Multiple sequence alignments and phylogenetic tree analysis indicated that CyHV-3 vIL-10 has low homology with vIL-10 from other viruses or IL-10 from its host (common carp), such that its evolutionary origins remain unclear. But structural simulations of CyHV-3 vIL-10 showed a very similar tertiary structure to the reported vIL-10 and its host suggesting that vIL-10 may have similar functionality to other virus-encoded vIL-10 or IL-10, at least in carp IL-10. Upregulation of *socs3* on carp leukocytes with CyHV-3 vIL-10 stimulation confirmed the conservative signaling pathway among mammalian IL-10, carp IL-10

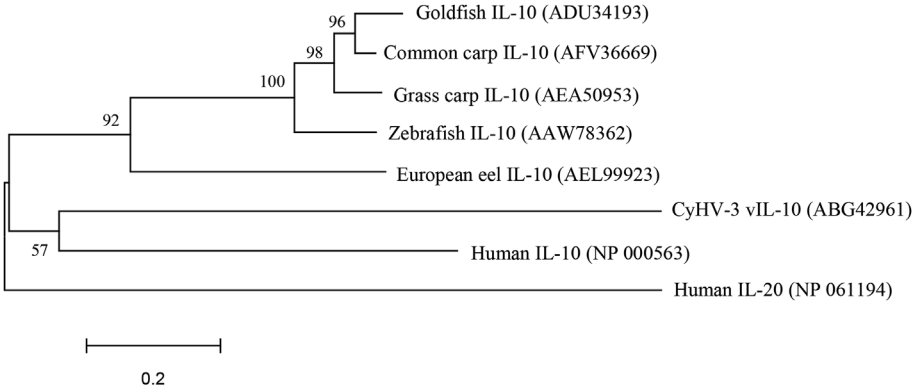

Fig. 6. Phylogenetic tree depicting the relationship among CyHV-3 vIL-10 gene, fish IL-10 genes, human IL-10 gene and human IL-20 gene inferred by the neighbor-joining method using the Mega 6.0. Reliability of the branching is indicated at the nodes as the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates).

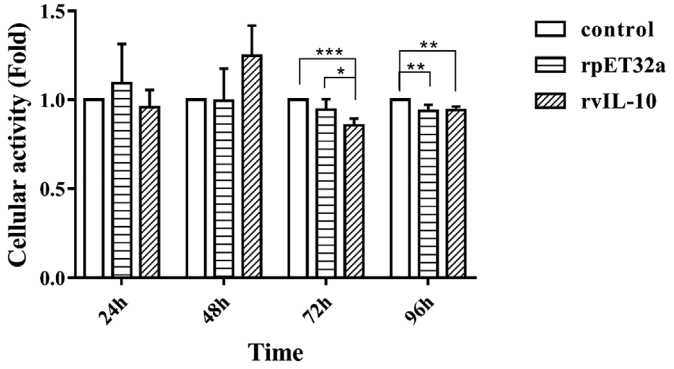

Fig. 7. The effect of CyHV-3 vIL-10 on cellular activity of EPC cells. Data are mean + SD of n = 5. Asterisks (*) indicates the significant differences between two groups. * indicate P < 0.05, ** indicate P < 0.01, ***indicate P < 0.001.

and CyHV-3 vIL-10 [1,14,23]. Both carp IL-10 and CyHV-3 vIL-10 activate the same signaling pathway in carp leukocytes which suggests a functional conservation of these two molecules [14]. There was a report that grass carp IL-10 can enhance PBLs cell viability after 72 h stimulation [24]. However, CyHV-3 vIL-10 reduces EPC cell viability at 72 h, which is different from the grass carp IL-10 [24].

The most definitive and conservative function of vIL-10 is to inhibit or downregulate the expression of immune-related genes. HCMV cmvIL-10 and RhCMV vIL-10 have been shown to inhibit LPS-stimulated production of pro-inflammatory cytokines on peripheral blood mononuclear cells (PBMCs) and monocytes [25–28]. EBV deleted for BCRF1 that encode vIL-10 infected human PBMCs produced significantly higher levels of IFN-γ, IL-2, IL-6 and TNF-β suggesting regulation of expression by EBV vIL-10 on host cells indirectly [29]. In addition, inhibition of cytokine synthesis has also been reported in ORFV vIL-10 and OvHV-2 vIL-10. OvHV2 vIL-10 can inhibit IL-8 expression, and ORFV vIL-10 inhibits IL-8, IL-1β, TNF-α and IFN-γ expression [30–34]. Therefore, the expression of immune-related genes is also the focus of this paper. In just stimulation with CyHV-3 vIL-10, there was no effect on gene expression. It is worth noting that high concentration of vIL-10 (10 μg/mL) stimulation can cause large expression of host IL-10. HCMV encoded human IL-10 homologue could amplifies its immunomodulatory by upregulating human IL-10 in monocytes [6]. While an increased production of human IL-10 was observed in human PBMCs infected with the BCRF1-deleted EBV [29]. These observations pointed to the diverse regulation of host IL-10 by vIL-10. CyHV-3 vIL-10 strongly inhibited the LPS-induced gene expression of TNF-α and INOS in macrophages which is similar to previous research [14]. Whereas an interesting, downregulation of TNF-α and INOS were also found in carp IL-10 stimulation [1]. Although strongly inhibition of the LPS-induced gene expression of IL-1β in

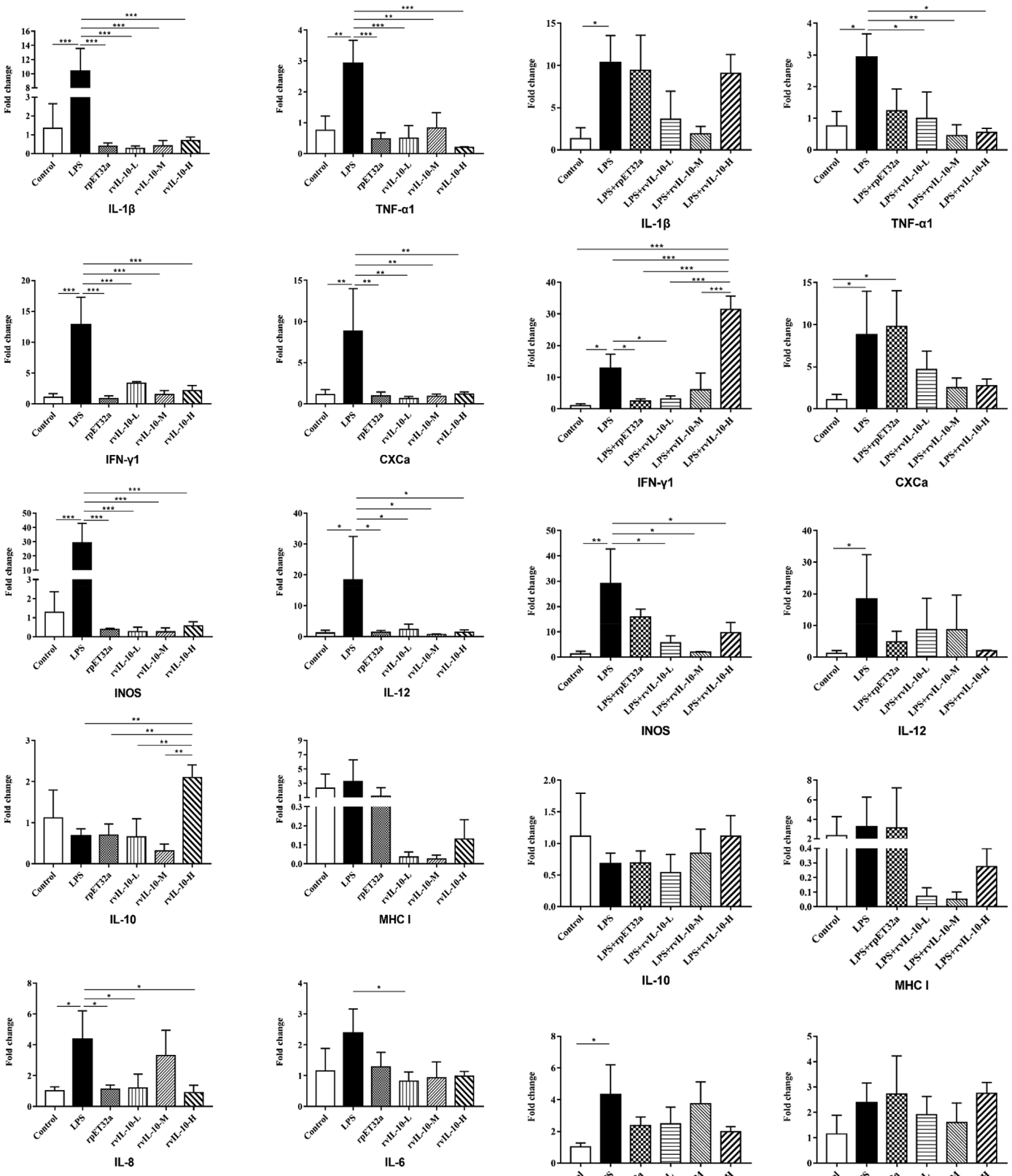

Fig. 8. CyHV-3 vIL-10 cannot regulate the expression of immune-related gene directly. Macrophages were stimulated for 3 h with increased concentrations of vIL-10 (rvIL-10-L, 0.1 μg/mL rvIL-10; rvIL-10-M, 1 μg/mL rvIL-10; rvIL-10-H, 10 μg/mL rvIL-10). LPS (20 μg/mL) as positive control and tag protein (rpET32a) as blank control. Gene expression was normalized relative to 40s and β-actin as reference genes. Data are mean +SD of n = 3 fish for macrophages. Asterisks (*) indicates the significant differences between two groups. * indicate $P < 0.05$, ** indicate $P < 0.01$, ***indicate $P < 0.001$.

(caption on next page)

Fig. 9. CyHV-3 vIL-10 downregulates the expression of some immune-related gene stimulated by LPS. Macrophages were stimulated for 3 h with LPS (20 μg/mL) in combination with increased concentrations of vIL-10. LPS alone as positive control and LPS in combination with tag protein (rpET32a) as blank control. Gene expression was normalized relative to 40s and β-actin as reference genes. Data are mean +SD of n = 3 fish for macrophages. Asterisks (*) indicates the significant differences between two groups. * indicate $P < 0.05$, ** indicate $P < 0.01$, ***indicate $P < 0.001$.

macrophages stimulated with carp IL-10, CyHV-3 vIL-10 has a downward trend in IL-1β which is not significantly [1]. The thought-provoking fact is that low concentrated vIL-10 inhibited IFN-γ1 expression while high concentrated vIL-10 significantly upregulated its expression unexpectedly. Similar results have been reported previously. Piazzon et al. found that no significant upregulation of IFN-γ gene expression was observed in macrophages and neutrophils stimulated with LPS, but when stimulated with LPS in the presence of carp IL-10, upregulation trend of IFN-γ was found in neutrophils [1]. Different from EBV, HCMV and RhCMV, CyHV-3 vIL-10 has no significant down-regulation of MHC I expression, but a slight inhibition expression [35–37]. However, in Piazzon et al. studies, they found CyHV-3 vIL-10 does not affect MHC I expression but carp IL-10 inhibited the LPS-induced gene expression of MHC I strongly [1,14]. Although not statistically significant, the low expression of CXCa and IL-12 were observed with CyHV-3 vIL-10 stimulation. However, carp IL-10 strongly inhibited IL-12 expression [1]. Unlike previous studies, CyHV-3 vIL-10 does not affect IL-8 and IL-6 [1,14,29–31]. Altogether, these data suggest that CyHV-3 vIL-10 downregulates the expression of proinflammatory and MHC I gene in macrophages of common carp.

The present study was devoted to CyHV-3 ORF134 encoding an IL-10 homologue. It is confirmed that CyHV-3 vIL-10 not only has similar biological functions as other viruses encoding IL-10 homologue but also with common carp IL-10 *in vitro*. Further, it is verified that CyHV-3 vIL-10 evades immune clearance by mimicking host IL-10 function to establish a persistent infection.

Competing of interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.048.

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