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Immersion immunization with recombinant baculoviruses displaying cyprinid herpesvirus 2 membrane proteins induced protective immunity in gibel carp

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ABSTRACT

Cyprinid herpesvirus 2 (CyHV-2) is the causative pathogen of herpesviral haematopoietic necrosis disease, which has caused huge economic losses to aquaculture industry in China. In this study, nine truncated CyHV-2 membrane glycoproteins (ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146) and a GFP reporter protein were respectively expressed using baculovirus surface displaying system. Western blot showed that the proteins were successfully packaged in the recombinant virus particles. In baculovirus transduced gibel carp kidney cells, the target proteins were expressed and displayed on the fish cell surface. Healthy gibel carp were immunized by immersion with the recombinant baculoviruses and the fish treated with phosphate-buffered saline (PBS) were served as mock group. The expression of *interleukin-11 (IL-11)*, *interferon α (IFNα)* and a complement component gene *C3* were significantly up-regulated in most experimental groups, and *interferon γ (IFNγ)* expression in some groups were also induced after immunization. Subsequently, the immunized gibel carp were challenged by intraperitoneal injection of CyHV-2 virus. All the immunized groups exhibited reduced mortality after CyHV-2 challenge. In the groups immunized with baculoviruses displaying and expressing ORF25, ORF25C and ORF146, the relative percentage survival values reached 83.3%, 87.5% and 70.8%, respectively. Our data suggested that baculovirus-displayed ORF25, ORF25C and ORF146 could be potential vaccine candidates for the prevention of CyHV-2 infection in gibel carp.

1. Introduction

Cyprinid herpesvirus 2 (CyHV-2), also named as herpesviral haematopoietic necrosis virus (GHNV) or goldfish haematopoietic necrosis virus (GFHNV), belongs to the family *Alloherpesviridae* genus *Cyprinivirus* which includes carp pox virus (CyHV-1), CyHV-2 and koi herpesvirus (CyHV-3) [1,2]. Gibel carp (*Carassius auratus gibelio*) in all sizes are sensitive to the infection and the resulting disease caused by CyHV-2 during spring and autumn, especially when the water temperature is between 15 and 25 °C, the mortality rate can reach high up to 100%. Fish infected with CyHV-2 have typical symptoms such as lethargy, anorexia, necrosis of head kidney, eye protrusion, pale gills, kidney and spleen enlargement etc. [3]. The disease outbreaks have been reported in Japan [4], United States [5], Australia [6], UK [7] and China [3], and have brought serious economic losses to the carp aquaculture worldwide [3].

Observed by electron microscopy, the nucleocapsid of the CyHV-2 virion is hexagonal, with a diameter of 110–120 nm without envelope and 170–200 nm with envelope [3]. CyHV-2 is a double-stranded DNA virus and the genome is 290 kbp in length, flanked by inverted repeats at both the ends. The genome is predicted to contain 150 unique, functional protein-coding genes, of which four are duplicated in the terminal repeats. A CyHV-2 isolate (GenBank accession number: JQ815364) has been sequenced, the genome structure and composition have been analyzed, and 36 open reading frames (ORFs) are predicted to code for membrane proteins [1].

CyHV-2 infection can activate complex immune responses in crucian carp. miRNA expression profiles were analyzed via high-throughput sequencing of the kidney tissue of *Carassius auratus gibelio* infected with CyHV-2, and the study demonstrated that the differentially expression of miRNAs were involved in the regulation of various immune-related signaling pathways, including chemokine signaling

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pathways and MAPK pathway [8]. Using suppression subtractive hybridization and quantitative PCR detection, 11 immune-related genes, especially *nfkbiab*, *IL-11* and *IL-10R*, were significantly upregulated in the moribund crucian carp infected with CyHV-2 [9]. In a recent report, combined transcriptomic/proteomic analysis of *Carassius auratus gibelio* in CyHV-2 infection revealed that the herpes simplex infection pathway, RIG-I-like receptor signaling pathway, necrotic pathway and p53 signaling pathway were activated, and the phagosome pathway was suppressed after CyHV-2 infection [10].

Vaccination is normally a cost-effective way to prevent viral infectious diseases. However, only a few vaccine researches on the prevention of the disease caused by CyHV-2 have been reported. It turned out that formalin inactivated CyHV-2 vaccine only provided about 50% protection in goldfish *Carassius auratus*, and a booster shot could improve the immune responses and the relative percentage survival (RPS) values increased to 63.6% [11]. In gibel carp, it was reported that immunization via the intraperitoneal injection of β -propiolactone inactivated vaccine effectively activated the innate and adaptive immunity and provided a relative survival rate of 71.4% after CyHV-2 challenge [12]. For the development of subunit vaccines, the CyHV-2 envelope glycoproteins of ORF25 family have been expressed and purified using yeast expression system. The protein immunization effectively induced protection immunity against CyHV-2 infection in gibel carp, suggesting the potential use of the membrane proteins as candidate vaccines [13].

Baculovirus is a double-stranded DNA virus with a genome size of 80–180 kbp, and it can only infect arthropods. Autographa californica Nuclear Polyhedrosis Virus (AcMNPV) is a model and the most studied species of baculovirus. Nucleic acid hybridization have shown that AcMNPV can enter into the cytoplasm and the nucleus of nonpermissive cell lines as effective as in the permissive cells, and the promoter elements of baculovirus immediate-early genes are functional in nonpermissive cells, although it cannot be replicated in the nonpermissive cells [14,15]. Since baculovirus can induce high level of humoral and cellular immunity, has low cytotoxicity and is biological safe for mammals, it has attracted a wide range of attentions as a vaccine vector [16–18]. Exogenous proteins can be displayed on the surface of baculovirus by fusion expression with the baculovirus envelope protein GP64 or other anchoring sequences [19], and the recombinant viruses can be used to transduce many types of animal cells including fish cells [20–22] and produce antibodies against the displayed antigen in animals [23–26]. AcMNPV can also be used as an adjuvant to enhance the immune effect of vaccines [27].

To evaluate the potential use of baculovirus displaying system in the prevention of CyHV-2 caused disease in gibel carp, nine type I membrane glycoproteins (ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146) were expressed and displayed on the surface of AcMNPV. Gibel carp were immunized by immersion with the recombinant baculoviruses and then challenged with CyHV-2. The immune responses and protection immunity against CyHV-2 induced by the immunization of the recombinant baculoviruses were investigated in this study.

2. Materials and methods

2.1. Ethics statement

Animal treatment in this study was carried out in accordance with the “Regulations For The Administration Of Affairs Concerning Experimental Animals”.

2.2. Gibel carp

Healthy gibel carp (body weight 5.5 ± 0.5 g) were purchased from the Renliuliangyou fishing farm in Lintong District, Shaanxi Province. The fish was cultivated in 160 L tanks at water temperature of 25 °C, fed

with commercial foods twice a day, and kept for 2 weeks to adapt to the laboratory environment before the experiment.

2.3. Viruses and cells

The tissue samples of CyHV-2 infected gibel carp used in this study were gifted by professor Chengliang Gong at Soochow University, and Dr. Yuan Junfa at Huazhong Agricultural University. *Spodoptera frugiperda* 9 cells (Sf9) were cultured in our laboratory as described previously [28].

The gibel carp kidney cell (GiCK) was prepared in our laboratory using the following method. Gibel carp were cultivated in sterilized water for 4 h before the experiment. The kidney of fish was dissected and chopped aseptically, then washed twice with M199 medium (Basalmedia Technologies, China) supplemented with 1000 mg/mL streptomycin, 1000 U/mL penicillin, 25 μ g/mL amphotericin B and 250 μ g/mL gentamicin. The tissue was dispersed into single cell suspension in M199 medium supplemented with 10% fetal bovine serum (FBS) after digestion with 0.25% trypsin solution. The suspension was then filtrated with a 100 μ m cell strainer, and the cells were pelleted by centrifugation at $200 \times g$ for 10 min and then resuspended with M199 medium supplemented with 20% FBS, 200 mg/mL streptomycin, 200 U/mL penicillin, 5 μ g/mL amphotericin B and 50 μ g/mL gentamicin. The cells were incubated at 28 °C, and half of the medium was replaced with fresh medium every three days. After the formation of cell monolayer, the cells were digested with 0.25% trypsin and subcultured every 6–8 days. After 10 passages, the concentration of FBS was reduced from 20% to 10% and antibiotics were also reduced to contain 100 mg/mL streptomycin, 100 U/mL penicillin, 0.25 μ g/mL amphotericin B and 50 μ g/mL gentamicin.

2.4. Generation of recombinant baculoviruses displaying CyHV-2 membrane proteins

2.4.1. Amplification of the genes for CyHV-2 membrane proteins

The total DNA was extracted from the homogenized tissue, using phenol/chloroform extraction method. By bioinformatics analysis of the CyHV-2 gene sequences, ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146 were selected as the target genes, and their signal peptides, ectodomains, transmembrane domains, and endodomains, were respectively predicted by SignalP server (<http://www.cbs.dtu.dk/Services/SignalP/>) and TMHMM Server (<http://www.cbs.dtu.dk/services/TMHMM/>). The gene sequences for the predicted ectodomains were amplified by PCR, using the specific primers listed in Table 1.

2.4.2. Plasmid construction

The plasmid pQBD (Bacmid Ltd. Shaanxi, China) was digested with restriction enzymes *Bam*HI and *Eco*RI. Truncated gene fragments (ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146) were then cloned into the linearized pQBD using ClonExpress II One Step Cloning Kit (Vazyme, China) such that these gene fragments could be expressed as fusion proteins, containing an N-terminal AcMNPV gp64 signal peptide (SP) and a C-terminal AcMNPV gp64 transmembrane domain (TM), under the control of chicken β -actin promoter (Fig. 1). The constructed recombinant plasmids were named as pQBD-ORF25, pQBD-ORF25C, pQBD-ORF25D, pQBD-ORF30, pQBD-ORF124, pQBD-ORF131, pQBD-ORF136, pQBD-ORF 142A, pQBD-ORF146, respectively. GFP was cloned into pQBD to generate pQBD-GFP, and it was also expressed as a fusion protein with the AcMNPV gp64 SP and TM and used as a negative control.

2.4.3. Construction of recombinant baculoviruses

Sf9 cells were seeded into 12-well plates to reach 50%–60% confluency. The linearized AcMNPV bacmid qBac-III (Bacmid Ltd. Shaanxi, China) and tranfer vectors carrying the target genes (pQBD-ORF25,

Table 1
Primers used for the amplification of the target genes coding for CyHV-2 membrane proteins.

Name	Primers ^a 5'-3'
CH25-F	acgatgacgacaaggatccAATACAACAACACCACCTACTAC
CH25-R	acatgaccaaacatgaatcCGTGAGGGTGGGAGACGTATC
CH25C-F	acgatgacgacaaggatccTTTGAGCCACTCTGAAGCCC
CH25C-R	acatgaccaaacatgaatcGGCTGTGATGCTGGTTCTGTCC
CH25D-F	acgatgacgacaaggatccTTCTAGTACCCGTGAACCAAC
CH25D-R	acatgaccaaacatgaatcAAGCAGCGACACGGTTTGTTCG
CH30-F	acgatgacgacaaggatccGACTGGTCCACTGTCTGTGCCCC
CH30-R	acatgaccaaacatgaatcCCATGTGTACTCCGAGTCTGTAT
CH124-F	acgatgacgacaaggatccACTCTGACTACTATGGATGATT
CH124-R	acatgaccaaacatgaatcATCCATGATGTAAGTGTCCAAG
CH131-F	acgatgacgacaaggatccCTGAACGGCACTAAAACCAAG
CH131-R	acatgaccaaacatgaatcCGCGAACGGCCCTTGGCGCAAG
CH136-F	acgatgacgacaaggatccACCAATGCCACTGTACACCTG
CH136-R	acatgaccaaacatgaatcGCCTTGGCGCAACTCACCAG
CH142A-F	acgatgacgacaaggatccCCCATACCAACAACCAGACCTC
CH142A-R	acatgaccaaacatgaatcCCTGCCCTCAATCAATTTAGCC
CH146-F	acgatgacgacaaggatccCACCGAATATTACCAACAACAAC
CH146-R	acatgaccaaacatgaatcCTCGTCTCGGTGTCTGACTCGC
GFP-F	acgatgacgacaaggatccGTGAGCAAGGGCGAGGAGCTGT
GFP-R	acatgaccaaacatgaatcCTGTACAGCTCGTCCATGCCG

^a Lowercase letters indicate the sequences for restriction enzyme sites and their protective bases.

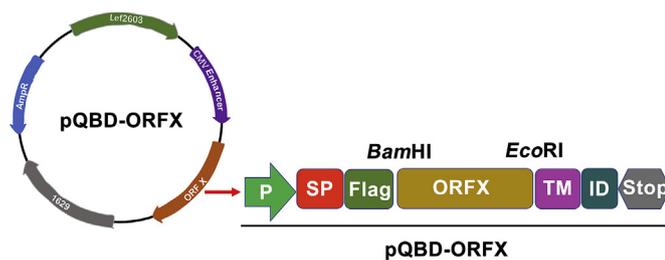


Fig. 1. Strategy for the construction of baculovirus vectors. P: Chicken β -actin promoter, SP: Autographa californica nuclear polyhedrosis virus (AcMNPV) gp64 signal sequence, TM: AcMNPV gp64 transmembrane domain, ID: AcMNPV gp64 inner domain.

pQBD-ORF25C, pQBD-ORF25D, pQBD-ORF30, pQBD-ORF124, pQBD-ORF131, pQBD-ORF136, pQBD-ORF142A, pQBD-ORF146 and pQBD-GFP were co-transfected in pairs into Sf9 cells. After cultured in SFX insect medium (HyClone, GE healthcare life science) for 6 days at 28 °C, the cell culture supernatant was collected. The recombinant baculoviruses were named as rAcMNPV-ORF25, rAcMNPV-ORF25C, rAcMNPV-ORF25D, rAcMNPV-ORF30, rAcMNPV-ORF124, rAcMNPV-ORF131, rAcMNPV-ORF136, rAcMNPV-ORF142A, rAcMNPV-ORF146, rAcMNPV-GFP, accordingly.

2.5. Detection of the expression and surface displaying of the fusion proteins

2.5.1. Western blot

To examine the protein expression, Sf9 cells were seeded into 12-well plates, and infected with the recombinant baculoviruses at a multiplicity of infection (MOI) of 3. The infected cells were maintained in SFX insect medium at 28 °C for 6 days, collected and washed three times with phosphate-buffered saline (PBS), and then lysed with 1 × SDS (sodium dodecyl sulfate) loading buffer for Western blot analysis (lysate from 1 × 10⁴ cells was loaded per well).

To detect the proteins displayed on the baculovirus virions, the supernatant of the infected cell culture was collected to harvest the recombinant baculoviruses by ultracentrifugation through a 1 ml 20% sucrose cushion at 100000 × g for 2 h. The precipitate in the centrifuge tubes was collected for Western blot analysis (about 1 × 10⁸ virions were lysed and loaded per well).

For Western blot analysis, proteins denatured with 1 × SDS loading buffer were resolved with 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membrane. After washing with tris-buffered saline with tween 20 (TBST), the membrane was blocked with 5% nonfat dry milk at 37 °C for 1 h, and immunoblots were developed using an anti-Flag monoclonal antibody at a dilution of 1:2000 as the primary antibody (CoWin Bio-tech, China) and a goat anti-mouse antibody at a dilution of 1:4000 as the secondary antibody (CoWin Bio-tech, China).

2.5.2. Immunofluorescence assay

To detect the recombinant proteins displayed on the cell surface, Sf9 cells were seeded into 12-well plates to reach 50% confluency and infected with the recombinant baculoviruses at an MOI of 1. The infected cells on cover slips were maintained in SFX insect medium at 28 °C for 4 d, collected and washed three times with PBS, and then fixed in 4% paraformaldehyde at room temperature for 15 min. After three washes with PBS, the cells were blocked with 10% Fetal bovine serum (FBS) for 1 h, and then sequentially probed with the anti-Flag antibody at a dilution of 1:100 and an FITC-conjugated anti-mouse IgG (CoWin Bio-tech, China) at a dilution of 1:100. The cells were then washed three times with PBS, and mounted with mounting medium, and the cover slips were sealed on slides using nail polish. The immunofluorescence was examined under an inverted fluorescence microscope (Lecia DMI8, Lecia).

2.5.3. Transduction of GiCK cells

The GiCK cells were seeded into a 35 mm petri dish to reach 50% confluency. Recombinant baculovirus rAcMNPV-GFP was added onto the cell monolayer at an MOI of 100, incubated for 4 h at 28 °C, and then the supernatant was removed and replaced with fresh M199 medium. After incubation for 48 h, the expression of the GFP was examined under the inverted fluorescence microscope.

2.5.4. Flow cytometry

To detect the displaying of the CyHV-2 membrane proteins on the baculovirus infected Sf9 cells and baculovirus transduced GiCK cells, the infected/transduced cells were scraped off and collected by centrifugation at 1000 × g for 5 min. After wash with PBS, the non-permeabilized cells were blocked with 10% FBS and then stained with the anti-Flag antibody and FITC-conjugated secondary antibody as described in 2.5.2. For the detection of protein expression in baculovirus transduced GiCK cells, the cells were also fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 at room temperature for 10 min, and then stained with the antibodies. Flow cytometry data were collected using an ACEA NovoCyte (ACEA Biosciences Inc.) at an excitation wavelength of 488 nm. The data analysis was performed using a NovoExpress 1.3.0 software.

2.6. Immune responses in gibel carp

2.6.1. Vaccination

To evaluate the immunogenicity of the recombinant baculoviruses, specific pathogen free gibel carp were randomly divided into 11 groups (60 animals/group). 30 mL of recombinant baculoviruses rAcMNPV-ORF25, rAcMNPV-ORF25C, rAcMNPV-ORF25D, rAcMNPV-ORF30, rAcMNPV-ORF124, rAcMNPV-ORF131, rAcMNPV-ORF136, rAcMNPV-ORF142A, rAcMNPV-ORF146 and rAcMNPV-GFP (at a dose of 6 × 10⁵ TCID₅₀/mL) were diluted into 5 L freshwater, and gibel carp were immersed in the tanks for 2 h. The mock group was given the same volume of PBS. The fish were kept in 160 L water tanks and the temperature was maintained at 25 °C.

2.6.2. Detection of the transcription level of immune genes

Fish gills (3 animals/group) were collected at 1, 2, 4, 7, 15 days after immunization. The total RNA was isolated from the gills using

Table 2
Primers used in real-time PCR in this study.

Name	Primers 5'-3'
β -actinF	CTGTCTTCCATCCTCGTGGG
β -actinR	AGCCTCATCACCAACGTAGC
IL-11-F	TGACCAATCAGACCAGACACC
IL-11-R	CGCTGGAAGGAACTTGAACC
C3-F	AGTGAAATGGTGAAGCAGAAAAG
C3-R	TACGTATACCGAGACATCGAAGG
IFN α -F	TTGCAGATGGCTCGACAGAT
IFN α -R	GTCCACTCATTTCGGAAGC
IFN γ -F	TCCCTGAGAACCTGGACAAGA
IFN γ -R	TGGGTCATCTTCCTTGATCGC

RNAprep Pure Tissue Kit (TIANGEN, China) according to the manufacturer's instructions, and mRNA was reverse transcribed into cDNA using EasyScript®First-Strand cDNA Synthesis SuperMix (TRANS, China). Quantitative real-time PCRs were performed using ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme, China), and the expression levels of immune genes *Interleukin-11* (IL-11, GenBank No.: KC969198), component C3 (C3, GenBank No.: KF110786), *interferon alpha* (IFN α , GenBank No.: MK093763.1) and *interferon gamma* (IFN γ , GenBank No.: EU909368.1) were detected using the primers listed in Table 2. The expression levels of the immune genes were analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$) with β -actin gene as an internal control [29].

2.7. Challenge test

At 47 days post-immunization, 50 μ l tissue homogenate of kidney from CyHV-2 infected gibel carp was intraperitoneally injected into each fish in the eleven groups. The mortalities were recorded daily for 30 days after the challenge. DNA was extracted from dead fish and CyHV-2 infection was verified by PCR detection of the ORF79 gene coding for the viral DNA polymerase catalytic subunit [1] using primers ORF79-F (5'-CATCAGCCAGAGTCCATAGTGTG-3') and ORF79-R (5'-GTTTCAGATTACCAGGATGCGTTG-3'). The relative percentage survival (RPS) values were calculated using the following equation [30]:

$$RPS = (1 - \text{percent mortality in vaccinated fish} / \text{percent mortality in control fish}) \times 100\%$$

2.8. Statistical analysis

The results were expressed as mean \pm standard deviation (SD) and all statistical analyses were performed using the GraphPad Prism 7.0 and SPSS 24 software package. The statistical significance was calculated by Student's *t*-test.

3. Results

3.1. Generation of recombinant baculoviruses in Sf9 cells

To make transfer vectors for the generation of baculoviruses displaying CyHV-2 membrane proteins, truncated gene fragments (ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146, GFP) were cloned into pQBD between the *Bam*HI and *Eco*RI sites. The expression cassette for the displaying of the membrane proteins is illustrated in Fig. 1. The constructed plasmids were verified by PCR and sequencing analyses (TSINGKE, China). Linearized AcMNPV bacmid qBac-III was respectively co-transfected in pairs with the transfer vectors (pQBD-ORF25, pQBD-ORF25C, pQBD-ORF25D, pQBD-ORF30, pQBD-ORF124, pQBD-ORF131, pQBD-ORF136, pQBD-ORF142A, pQBD-ORF146, pQBD-GFP) into Sf9 cells, and the cell culture media containing the recombinant baculoviruses (rAcMNPV-ORF25, rAcMNPV-ORF25C, rAcMNPV-ORF25D, rAcMNPV-ORF30,

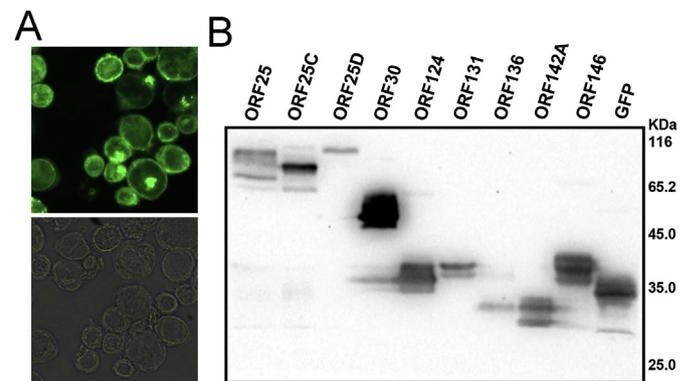


Fig. 2. Detection of the fusion protein expression in baculovirus infected Sf9 cells. (A) Observation of the GFP protein expression by Fluorescence microscopy. Sf9 cells were infected with recombinant baculovirus rAcMNPV-GFP at an MOI of 0.5, and the GFP expression was observed at 5 days post infection. (B) Detection of the expression of Flag-tagged proteins by Western blot. Sf9 cells were infected with the recombinant baculoviruses expressing the indicated CyHV-2 ORF and the whole cell lysates were collected at 5 days post infection for the protein detection.

rAcMNPV-ORF124, rAcMNPV-ORF131, rAcMNPV-ORF136, rAcMNPV-ORF142A, rAcMNPV-ORF146, rAcMNPV-GFP) were harvested when virus-induced cytopathic effect (CPE) appeared. The generation and infection of rAcMNPV-GFP in Sf9 cells was confirmed by the observation of the GFP expression under an inverted fluorescence microscope (Fig. 2A).

3.2. Expression and displaying of CyHV-2 membrane proteins by baculovirus expression system

To examine the protein expression, Sf9 cells infected with the recombinant baculoviruses were collected for Western blot analysis. The results showed that all the truncated CyHV-2 membrane proteins were successfully expressed in Sf9 cells (Fig. 2B). Exogenous proteins ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146 and GFP fused with the baculovirus envelope protein GP64 transmembrane domain and inner domain had predicted molecular weights of about 67, 66.9, 80.3, 31.9, 28.5, 36.7, 15.0, 14.3, 35.1 and 34 kDa, respectively. For most of the proteins, multiple bands, with some of them were obviously bigger than their predicted molecular weights, were detected (Fig. 2B). As all the membrane proteins have potential glycosylation sites, this result suggests that these CyHV-2 membrane proteins are expressed and post-translationally modified in Sf9 cells.

With a signal peptide and a transmembrane domain derived from baculovirus gp64 fused with the truncated CyHV-2 membrane proteins, all the proteins are postulated to be able to be anchored on the cell membrane. To verify the anchoring of these proteins on cell membrane, non-permeabilized cells were probed with anti-Flag antibody and FITC-conjugated secondary antibody. Observation under fluorescence microscope showed that all the CyHV-2 fusion proteins were well expressed and displayed on the insect cell surface (Fig. 3A). Flow cytometry analysis of the stained cells confirmed the expression of the recombinant proteins on the infected cell surface (Fig. 3B).

To examine whether the CyHV-2 proteins could be packaged in the baculovirus virions, baculovirus particles were purified by ultracentrifugation through a sucrose cushion. All the recombinant proteins were detected in the purified baculovirus particles by Western blot, indicating that these CyHV-2 membrane proteins were successfully displayed on the baculovirus virions (Fig. 3C).

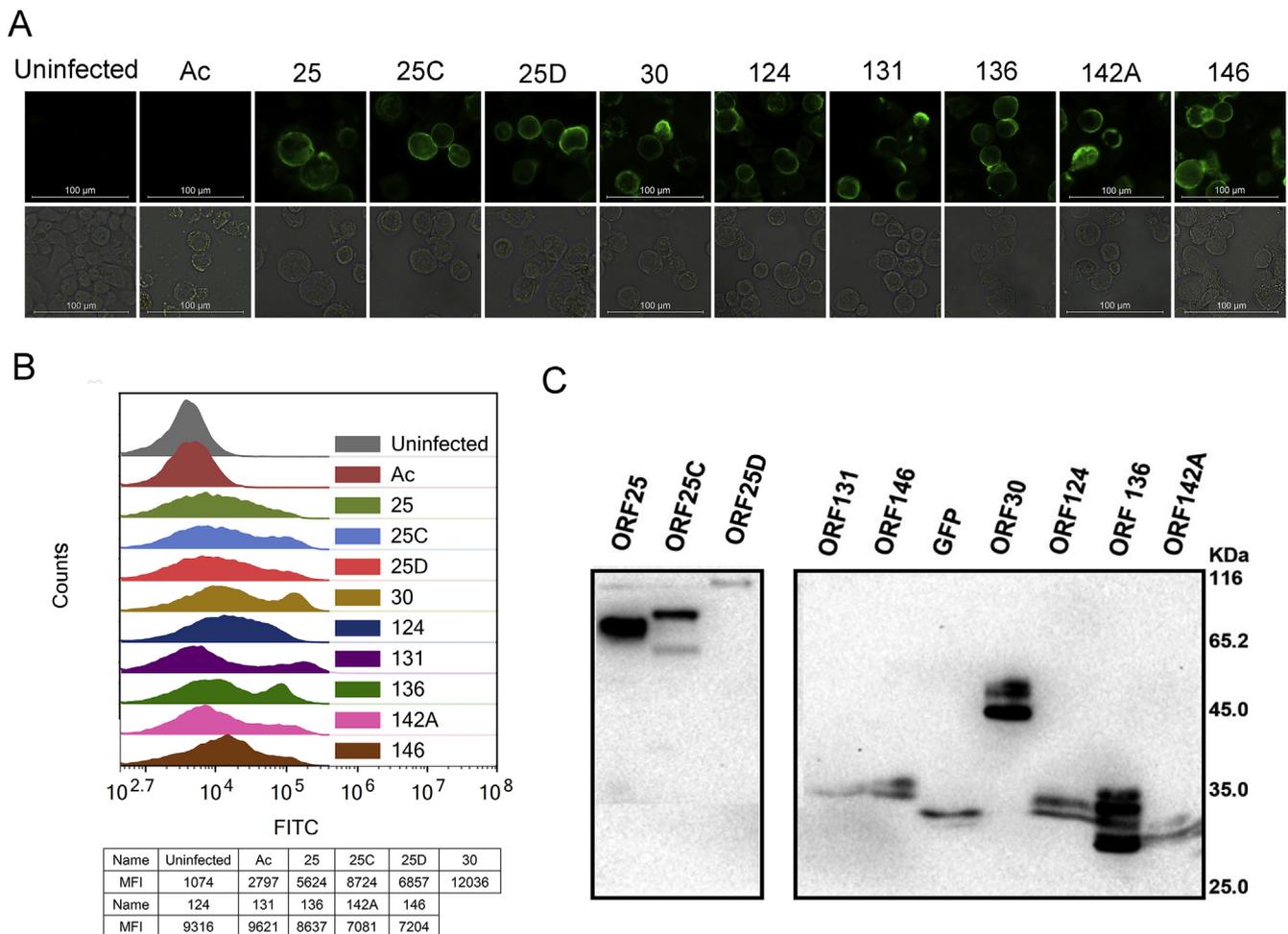


Fig. 3. Detection of the surface displaying of the CyHV-2 fusion proteins on baculovirus infected Sf9 cells and baculovirus particles. (A) Immunofluorescent observation of the protein expression on the cell surface. Sf9 cells were infected with the recombinant baculoviruses expressing the indicated CyHV-2 ORF, and the non-permeabilized cells were stained with anti-Flag antibody and FITC-conjugated secondary antibody. (B) Flow cytometry analysis of the baculovirus infected cells. The cells were also non-permeabilized and stained with the antibodies as described in A. The sample Ac in the figure indicates cells infected with a control AcMNPV that does not express Flag-tagged protein. (C) Recombinant baculovirus particles were purified by ultracentrifugation at 100000 × g for 2 h, and the displaying of CyHV-2 membrane proteins were determined by Western blotting.

3.3. Transduction of GiCK cells

To investigate whether the recombinant baculoviruses could enter gibel carp cells and whether the exogenous proteins could be expressed by baculovirus transduction, rAcMNPV-GFP was added onto GiCK cell monolayer at an MOI of 100, and the expression of GFP was observed by fluorescence microscopy and flow cytometry. As shown in Fig. 4A and B, GFP was expressed in some GiCK cells, demonstrating that recombinant baculovirus could effectively transduce GiCK cells and the foreign gene under the control of chicken β-actin promoter carried by the virus could be efficiently expressed in the fish cell.

The expression of CyHV-2 fusion proteins in baculovirus transduced GiCK cells were detected by anti-Flag antibody staining and flow cytometry. The results showed that most CyHV-2 fusion proteins were well expressed in the cells permeabilized by Triton X-100 (Fig. 4C). However, the mean fluorescence intensity (MFI) of the cells transduced by rAcMNPV-ORF25D was very close to the MFI of the control cells transduced by AcMNPV, indicating that the ORF25D fusion proteins were not effectively expressed in the GiCK cells or its N-terminal Flag-tag was not well exposed for the antibody binding.

To examine whether the fusion proteins were displayed on the baculovirus transduced cells, non-permeabilized GiCK cells were stained by the anti-Flag antibody and then analyzed by flow cytometry. The results showed that most of the CyHV-2 fusion proteins were detected

on the surface of GiCK cells (Fig. 4D). The MFI of the rAcMNPV-ORF25D transduced cells was still close to the rAcMNPV transduced control cells, consistent with the result from the permeabilized cells. Surprisingly, the MFI of the non-permeabilized rAcMNPV-ORF146 transduced cells was lower than most of the groups expressing CyHV-2 fusion proteins, although the permeabilized rAcMNPV-ORF146 transduced cells had the highest MFI. This result suggested that ORF146 fusion protein could be efficiently expressed in the baculovirus transduced fish cells but somehow the protein or at least the N-terminal Flag-tag was not well exposed on the surface of the transduced cells.

3.4. Vaccination induced immune-related gene expression

Fish gills (3 animals/group) from 10 baculovirus-vaccinated groups and a mock-vaccinated group were collected at 1, 2, 4, 7, 15 days post-vaccination. The total RNA was extracted and reverse transcribed into cDNA. The expression levels of the immune-related genes *IL-11*, *C3*, *IFNα* and *IFNγ* were detected by SYBR Green real-time PCR, and the gene expression kinetics were shown in Fig. 5.

In all the immunized groups except the group immunized with rAcMNPV-ORF25C, immune genes *IL-11* and *C3* were significantly upregulated at 1 day post-vaccination, compared with the mock immunized group (Fig. 5A and B). The immune gene expression levels were maintained higher in most of the immunized groups than the

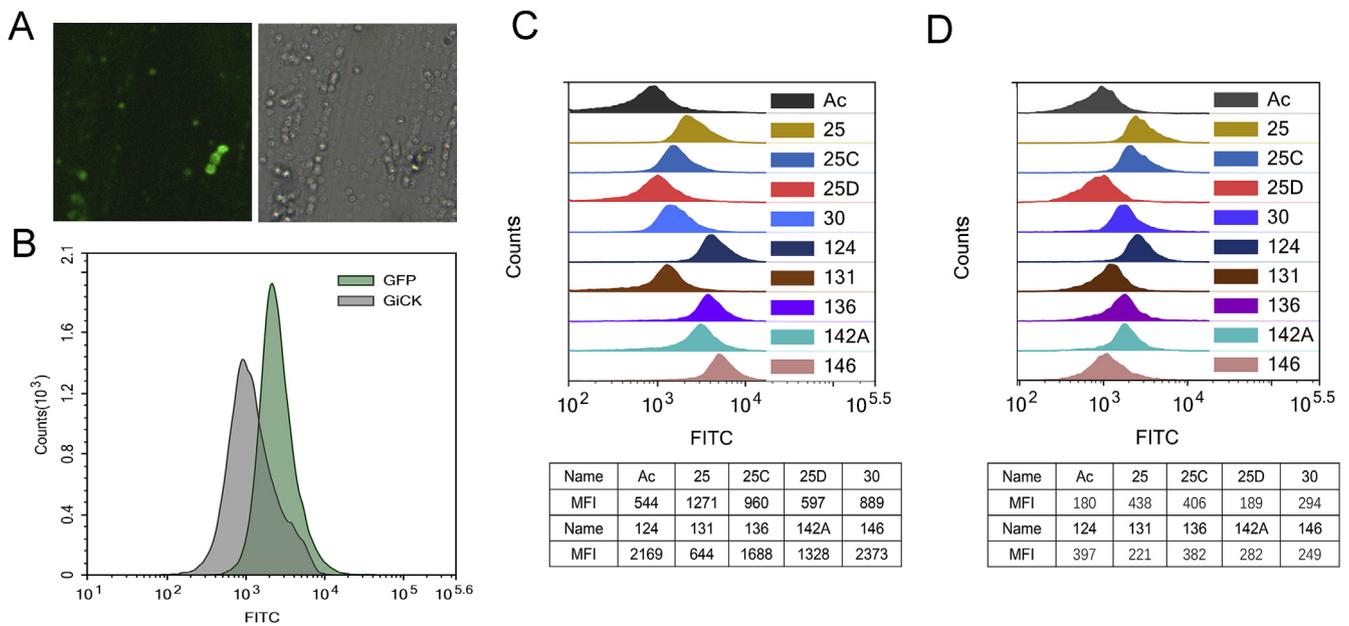


Fig. 4. Expression and surface displaying of the fusion proteins on baculovirus transduced Gibel carp kidney (GiCK) cells. (A) Fluorescence microscopy detection of the GFP expression. GiCK cells were transduced with rAcMNPV-GFP at an MOI of 100, and the GFP expression was observed at 2 days post transduction. (B) Flow cytometry detection of the GFP expression. (C) Detection of the expression of the CyHV-2 fusion proteins by flow cytometry. GiCK cells were transduced with the indicated baculoviruses at an MOI of 100, fixed and permeabilized at 2 days post transduction, and then stained with anti-Flag antibody and FITC-conjugated secondary antibody. (D) Detection of the surface displaying of the CyHV-2 fusion proteins by flow cytometry. The non-permeabilized GiCK cells were stained with the antibodies in parallel with the permeabilized cells. MFI:the mean fluorescence intensity.

mock group till 7 days post-vaccination for *C3* and 15 days post-vaccination for *IL-11*. Interestingly, the expression levels of *C3* showed a second peak at 4–7 days post-vaccination in some immunized groups. For the groups immunized with rAcMNPV-ORF25C and rAcMNPV-ORF25D, the *IL-11* and *C3* gene expression levels peaked at 7 days post-vaccination (Fig. 5A and B).

As baculovirus vaccination may mimic a virus infection which can induce the production of interferons (IFNs), the transcription of a type I IFN gene (*IFN α*) and a type II IFN gene (*IFN γ*) were also examined. At 1 day post-vaccination, rAcMNPV-ORF25, rAcMNPV-ORF30, rAcMNPV-ORF124, rAcMNPV-ORF131, rAcMNPV-ORF136 and rAcMNPV-ORF142A significantly triggered the *IFN α* expression, but the gene

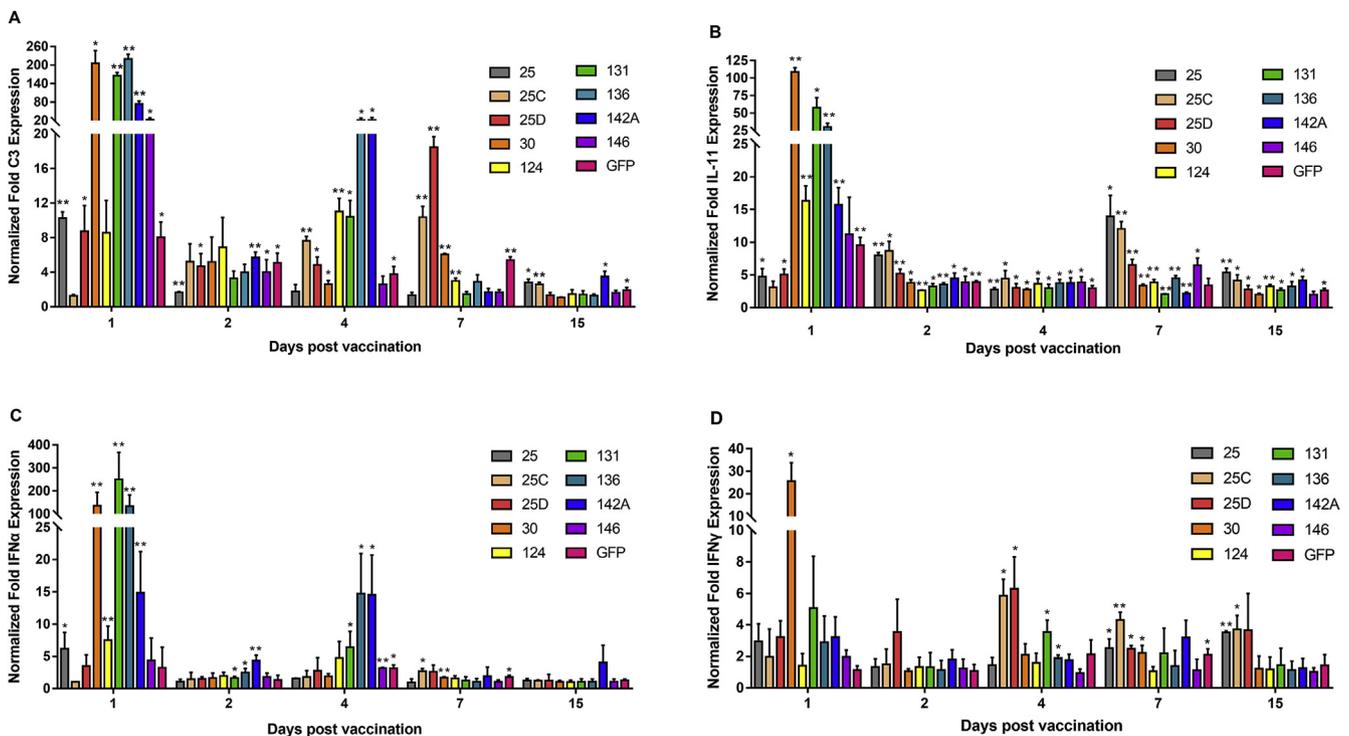


Fig. 5. The SYBR Green real-time PCR analysis of immune-related gene expression in the vaccinated groups. Gibel carps in the ten immunized groups were respectively immersed in tanks containing 30 mL of the indicated recombinant baculovirus and the mock vaccinated group received the same volume of PBS. At 1, 2, 4, 7, and 15 days post immunization, the expressions of *C3* (A), *IL-11* (B), *IFN α* (C) and *IFN γ* (D) in the gill were examined. (n = 3). *p < 0.05, **p < 0.01.

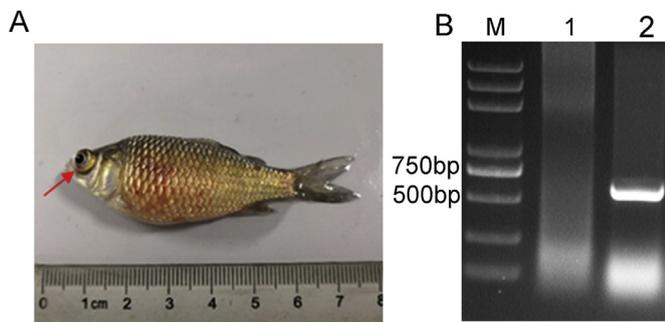


Fig. 6. Verification of the virus infection of Gibel carp injected with tissue homogenate containing CyHV-2. (A) A gibel carp that was infected with CyHV-2. (B) Detection of the CyHV-2 infection in the gibel carp by PCR using a pair of specific primers targeting ORF79. Lane 1: uninfected fish; Lane 2: Fish infected with CyHV-2.

expression levels quickly dropped at 2 days after vaccination. Similar as the *C3* gene, some vaccination groups showed a second *IFN α* expression peak at 4 days post-vaccination (Fig. 5C). For the *IFN γ* expression, only the rAcMNPV-ORF30 group was found to have significant higher expression level at 1 day post-vaccination. However, at days 4–15 after vaccination, some baculovirus vaccinations especially rAcMNPV-ORF25, rAcMNPV-ORF25C and rAcMNPV-ORF25D activated the *IFN γ* expression (Fig. 5D).

3.5. Protection of gibel carp against CyHV-2 challenge

To investigate whether the vaccination with recombinant baculoviruses could induce protective immunity in gibel carp against CyHV-2 infection, 50 μ l tissue homogenate from CyHV-2 infected fish was intraperitoneally injected into each fish in the eleven groups at 47 days post-immunization. In the mock-immunized group, the fish began to die at 4 days post challenge, and the dead fish showed typical clinical symptoms of CyHV-2 infection such as prominent eyeballs and congestion, abdominal swelling and bleeding, and darker colored dorsal fins (Fig. 6A). The infection of CyHV-2 was confirmed by PCR detection of the viral DNA (Fig. 6B). At 30 days post challenge, 24 out of 35 fish in the mock-immunized group died of virus infection (Table 3). All the groups immunized with recombinant baculoviruses including the rAcMNPV-GFP group showed higher survival rates after the CyHV-2 challenge than the mock-immunized group. Among them, rAcMNPV-ORF25C immunized group had the lowest cumulative mortality rate of 8.571%. The relative percentage survival (RPS) values of three groups (immunized with rAcMNPV-ORF25C, rAcMNPV-ORF25 and rAcMNPV-ORF146) were higher than 70% (Table 3, Fig. 7), suggesting that these recombinant baculoviruses had the potential to be developed as vaccines for the prevention of the outbreak of CyHV-2 caused disease in

Table 3

Cumulative percentage mortality (%) and calculated RPS values (%) following challenge with CyHV-2 in experimental groups of vaccinated fish by immersion immunization.

Immersion immunity	Mortalities (death/total)	RPS
rAcMNPV-ORF25	11.429% (4/35)	83.3%
rAcMNPV-ORF25C	8.571% (3/35)	87.5%
rAcMNPV-ORF25D	31.429% (11/35)	54.2%
rAcMNPV-ORF30	28.571% (10/35)	58.3%
rAcMNPV-ORF124	31.429% (11/35)	54.2%
rAcMNPV-ORF131	31.429% (11/35)	54.2%
rAcMNPV-ORF136	28.571% (10/35)	58.3%
rAcMNPV-ORF142A	62.857% (22/35)	8.3%
rAcMNPV-ORF146	20% (7/35)	70.8%
rAcMNPV-GFP	42.857% (15/35)	37.5%
PBS	68.571% (24/35)	—

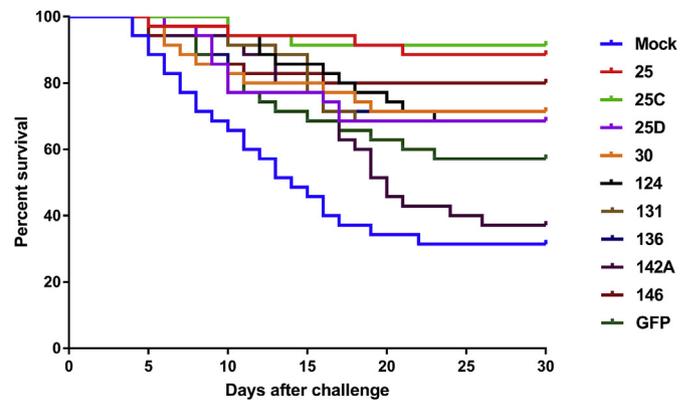


Fig. 7. Survival curves of gibel carp. The immunized gibel carps were challenged with CyHV-2 by intraperitoneal injection of 50 μ l of tissue homogenate per fish at 47 days post immunization. The mortality of each group (n = 35) was daily counted for 30 days.

gibel carp. Immunization with five recombinant baculoviruses displaying CyHV-2 membrane proteins (rAcMNPV-ORF25D, rAcMNPV-ORF30, rAcMNPV-ORF124, rAcMNPV-ORF131, rAcMNPV-ORF136) showed higher protection immunity than the rAcMNPV-GFP group and all their RPS values were higher than 50%, indicating that immunization with these five CyHV-2 membrane proteins could illicit protective responses in some degree in gibel fish against CyHV-2 infection. In the rAcMNPV-ORF142A group, the mortality rate was higher than that in the rAcMNPV-GFP group and close to the mock-immunized group, implying that ORF142A is not a suitable vaccine candidate for the prevention of CyHV-2 infection.

4. Discussion

Glycoproteins of enveloped viruses play key roles in viral attachment and entry, and they are often considered as the leading targets in vaccine design. CyHV-2 is the causative agent of herpesviral haematopoietic necrosis disease which has caused great economic losses to the aquaculture industry in China [3]. Although it is known that glycoproteins C (gC), gB, and gD are the major structural viral envelope components which are essential for herpesvirus attachment [31] and a previous study has shown that truncated ORF25, ORF25C and ORF25D produced from yeast expression system had antiviral effect on CyHV-2 *in vitro* and intramuscular inoculation of the proteins could induce protective immunity *in vivo* [13], it remains unclear which membrane proteins are involved in the entry of CyHV-2 into the host cell, and the antigenicity and immunogenicity of most of CyHV-2 membrane proteins remain to be investigated.

To search for potential vaccine candidates for the prevention of CyHV-2 caused disease, the ectodomains of nine of the viral type I membrane proteins including ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A and ORF146 were expressed on baculovirus surface, and the immunogenicity and protective efficacy induced by the baculovirus-displayed antigens were investigated in this study. Our data showed that eight of the membrane proteins, with the only exception of ORF142A, elicited better protection against the mortality caused by CyHV-2 infection than the control protein GFP. Especially, immunization with ORF25, ORF25C and ORF146 increased RPS rates to 83.3%, 87.5% and 70.8%, respectively. The group immunized with ORF25D had a RPS of 54.2%, similar as the previous immunization results by intraperitoneal injection of the protein purified from yeast expression system. With ORF25 and ORF25C immunized group showing better protection of 83.3% and 87.5%, our data here confirmed the previous report that ORF25, ORF25C and ORF25D could be potential candidate vaccines against CyHV-2 infection in gibel carp [13]. ORF25D is the biggest protein expressed in this study, its protein

level detected by Western blot was less than others in baculovirus infected Sf9 cells (Fig. 2B) and in purified baculovirus particles (Fig. 3C). In the rAcMNPV-ORF25D transduced GiCK cells, the expression of ORF25D was hardly detected (Fig. 4C and D). However, rAcMNPV-ORF25D immunization stimulated the expression of immune genes (especially $IFN\gamma$) (Fig. 5). We speculate that ORF25D was expressed in the baculovirus transduced fish cells but at a lower level than other CyHV-2 membrane proteins, and this may also explain why baculovirus delivered ORF25 and ORF25C exhibited higher protection (83.3% and 87.5% versus 75% and 63%) than the protein immunogens but baculovirus delivered ORF25D only provided a similar level of protection (54.2% versus 54%) as the purified protein [13].

Our study here also identified ORF146 as a good immunogen to illicit protective immunity against CyHV-2. By sequence comparison, the predicted membrane protein ORF146 from CyHV-2 and CyHV-3 share only about 30% amino acid sequence similarity (data not shown), and none of its homologous protein has been found in other herpesviruses. Flow cytometry analysis showed that the protein was well expressed but the protein, at least its N-terminus, was not well exposed in the baculovirus transduced GiCK cells (Fig. 4C and D). The role of ORF146 in the virus infection remains to be investigated.

Baculovirus expression system is a rapid and high yield protein production platform, which has been widely used in basic researches and pharmaceutical industry [32,33]. With the ability to efficiently transduce mammalian cells, baculovirus is also considered as a useful vector in gene therapy and vectored vaccine design [33,34]. It has been reported that recombinant baculoviruses can effectively transduce different types of fish cells and exogenous genes delivered by the virus transduction can be expressed in the transduced fish cells [20–22]. Immunization studies using baculovirus vectors expressing the VP6 protein of grass carp reovirus have revealed that baculovirus-vectored vaccine can induce immune responses in grass carp and have the potential to be developed as an orally administered vaccine [35,36].

In this study, we showed that recombinant baculovirus rAcMNPV-GFP could enter GiCK cells and the CyHV-2 fusion proteins under the control of a chicken β -actin promoter were successfully expressed in the transduced gibel carp cells (Fig. 4). After immersion immunization of the recombinant baculoviruses displaying and expressing CyHV-2 membrane proteins, the expression of immune genes of *IL-11* and *C3* were rapidly and dramatically upregulated at day 1 in most groups except the group immunized with rAcMNPV-ORF25C (Fig. 5). As it seemed that protein immunization in previous studies did not activate the immune genes so quickly [13], we postulate that it was the entry of the baculoviruses that induced the immune gene expression at 1 day post immunization. The upregulation of immune gene expression lasted at least for 7 days, and interestingly a second gene expression peak, which was probably contributed by the expression of CyHV-2 membrane proteins after the entry of recombinant baculovirus, appeared in some of the immunized groups at 4–7 days post immunization. We do not know why rAcMNPV-ORF25C failed to activate the immune genes expression at day 1, but it induced strong immune responses later and possessed the best protective potency in CyHV-2 challenge (Fig. 7). Further studies are needed to investigate whether the ORF25C displayed on baculovirus surface could interact with any fish molecules and delay the entry of the vector into fish tissues.

Cytokines are a group of small soluble proteins secreted by immune cells and tissues that play regulatory functions between cells. Interleukin-11 is an active participant in the immune responses of hosts to infections in cytokine networks, and it can act synergistically with other cytokines to stimulate proliferation and differentiation of early and late haematopoietic progenitor cells [37,38]. IL-11 can stimulate the production of several acute phase plasma proteins in hepatocytes and can also stimulate B cells to produce immunoglobulins. IL-11 presents in all fishes and is expressed in most salmon organs and tissues [39]. The complement system plays a crucial role in the host's innate defense and adaptive immunity against pathogenic challenges [40].

The third complement component (C3) is a key protein in the complement system [41] and bacterial infection can lead to significant changes in the expression of the *C3* gene in the immune tissue [42]. Complement C3 regulates the immune responses of B cells and T cells. Distinct C3 forms have been successfully purified from the serum of a single carp, demonstrating the presence of the C3 protein and suggesting that C3 plays a vital function in the defense responses of the bony fish [43]. In previous vaccination studies with β -propiolactone inactivated CyHV-2 or truncated proteins expressed in *P. pastoris*, quantitative PCR analysis revealed that the expression levels of *IL-11* and *C3* were significantly up-regulated in the immunized gibel carps [12,13]. Our data showed that the two immune genes could be rapidly activated within a day after the immunization of recombinant baculoviruses, implied their important role in the innate immunity of gibel fish.

Interferons (IFNs) are cytokines produced by many cell types in response to a viral infection. In fish such as crucian carp, $IFN\alpha$ (a type I IFN) has an ability to induce the expression of ISGs and also of itself by JAK-STAT signaling pathway [44]. The production of $IFN\alpha$ occurs very rapidly after virus infection, and it plays a role in the first line of defense against viruses [45,46]. The data in this study showed that the expression of $IFN\alpha$ was quickly triggered in most of the immunized groups, suggesting that the baculovirus entry could induce the innate immune response in gibel carp.

$IFN\gamma$ is a type II IFN which is produced by natural killer cells and T lymphocytes in response to MHC-presented antigens. In contrast to type I IFNs, $IFN\gamma$ promotes cell mediated immune responses to intracellular pathogens including viruses and has a key role in adaptive cell mediated immunity [46,47]. Our data showed that rAcMNPV-ORF25 and rAcMNPV-ORF25D immunizations significantly increased the $IFN\gamma$ expression, indicating that the T cell mediated adaptive immune responses might be activated, which may account for the high protective immunity induced by the two immunogens. Although rAcMNPV-ORF146 also greatly reduced the mortality caused by CyHV-2 infection, its vaccination did not significantly activate the $IFN\gamma$ expression. Besides T cell mediated immune responses, neutralizing antibody also plays a crucial role in providing protection against some viral diseases. Due to the lack of cell lines to passage CyHV-2, we did not examine the neutralizing antibodies elicited by the baculovirus-delivered antigens in this study. Further studies are needed to clarify how and why the membrane proteins used in this study provided different levels of protection against CyHV-2 caused disease in gibel carp.

5. Conclusion

The results of this study demonstrated the expression of CyHV-2 truncated membrane proteins (ORF25, ORF25C, ORF25D, ORF30, ORF124, ORF131, ORF136, ORF142A, ORF146) using baculovirus expression system, and the displaying of the viral membrane proteins on baculovirus surface as well as on the baculovirus-infected/transduced insect and fish cells. Simple immersion immunization of the recombinant baculoviruses induced protective immune responses in gibel carp against a subsequent CyHV-2 challenge. The RPS values in rAcMNPV-ORF25, rAcMNPV-ORF25C and rAcMNPV-ORF146 immunized group reached 83.3%, 87.5% and 70.8%, respectively, indicated their potential as vaccine candidates in the prevention of CyHV-2 caused disease.

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