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Characterization and functional analysis of a novel C1q domain-containing protein from grass carp (*Ctenopharyngodon idella*) in response to bacterial challenge

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ABSTRACT

C1q domain-containing (C1qDC) proteins are significant pattern recognition receptors in the innate immune system that play important roles in regulating immune responses in vertebrates and invertebrates. In this study, a gene encoding the fish C1qDC (designated *Ci*C1ql2) from the grass carp *Ctenopharyngodon idella* was characterized for the first time. Like other reported C1ql proteins, *Ci*C1ql2 contains a conserved C1q domain and a signal peptide at its 5′ end. Expression profile analysis revealed that *Ci*C1ql2 was mostly expressed in the intestine but was expressed at low levels in the brain and gill. *In vivo* injection experiments directly revealed that *Ci*C1ql2 exhibited strong responsiveness to *Aeromonas hydrophila*, *Aeromonas veronii* and pathogen-associated molecular pattern (PAMP) challenge, as indicated by the presence of lipopolysaccharide (LPS) and peptidoglycan (PGN), and that the expression of these genes was significantly upregulated in the intestine of *C. idella*. Additionally, the recombinant *Ci*C1ql2 protein obviously promoted the agglutination of the gram-negative bacteria *A. hydrophila* and *A. veronii* and the gram-positive bacterium *Bacillus subtilis*, as shown by *in vitro* binding experiments. Moreover, *Ci*C1ql2 protein injection experiment revealed that *Ci*C1ql2 significantly enhanced the expression levels of classic complement genes (C3, C5, and C7) and inflammatory cytokines (TNF-α, IL-1β, and IL-8), while knockdown of *Ci*C1ql2 decreased the expression of these immune-related genes. Taken together, our results suggested that *Ci*C1ql2 could act as a pathogenic pattern recognition receptor involved in complement activation and that this might be related to the intestinal defense of *C. idella* against bacterial infection.

1. Introduction

The complement system is an essential component of innate immunity and serves as a bridge between innate and adaptive immune responses (Nesargikar et al., 2012). The complement system consists of a cascade of soluble proteins, membrane-bound receptors, and regulatory factors that work together to recognize, modulate, and lyse pathogens, thereby mediating inflammatory responses that are triggered by pathogen invasion (Merle et al., 2015). There are three pathways in the complement system that contribute to immune defense: the classical pathway, the alternative pathway, and the lectin pathway. The complement component 1q, which has a typical C1q globular (gC1q) domain, was identified as an important recognition molecule and is responsible for recognizing and binding to antigen-antibody complexes that are formed by IgM or IgG antibodies in the classic complement pathway (Bally et al., 2019; Zong et al., 2019). Upon binding, C1r and C1s, which are proteases, are activated and cleave other complement components to initiate the cascade reaction (Kishore and Ghebrehiwet,

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2020; Vadászi et al., 2022). Subsequently, the activation of C2, C3, and C4 leads to the formation of a membrane attack complex (C5b-C9), resulting in elimination of the pathogen (Daha, 2010). This process demonstrates the role of C1q molecules in activating the classical complement pathway. C1q domain-containing proteins (C1qDCs) are further classified into three subfamilies, namely, the C1q, C1q-like, and ghC1q families, on the basis of their structural characteristics (Huang et al., 2016; Li et al., 2019). The C1qDC family is a large protein family, and its members possess a gC1q domain and are involved in comprehensive adaptation to ligands, such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), poly I:C, and gram-negative bacteria (Wang et al., 2015; Zhu et al., 2021; Zong et al., 2019). In addition, C1qDC family members participate in various immune responses, including activating the complement pathway (M.-f Li et al., 2023; J. Li et al., 2023), clearing retroviruses (Liang et al., 2022) and promoting cell adhesion (Wang et al., 2022).

An increasing number of C1qDC proteins have recently been identified in vertebrates and invertebrates. C1qDC functions as a pattern recognition receptor to participate in innate immune responses in *Lethenteron camtschaticum* (Pei et al., 2016) and the pacific oyster *Crassostrea gigas,* and it plays a vital role in specifically recognizing certain gram-negative bacteria and opsonins to enhance phagocytosis (Lv et al., 2018). The *Po*C1qDC protein from *Paralichthys olivaceus*, which has a collagen-like structure, is able to mediate complement activation during bacterium-induced inflammatory responses (M.-f Li et al., 2023; J. Li et al., 2023). In *Argopecten irradians*, *Ai*C1qDC can promote the agglutination of fungi and bacteria (Wang et al., 2012). C1qDC from a colonial ascidian is involved in the inflammatory response (Peronato et al., 2021). Based on the current research, C1qDC plays a significant role in the innate immune responses of aquatic animals.

Ctenopharyngodon idella, which belongs to the Cyprinidae family, is cultured mainly in China, Russia and Bulgaria and ranks among the top cultured fishes in terms of aquaculture yield. In recent years, bacterial enteritis has led to an increase in the mortality rate of *C. idella*, significant decreases in production, and substantial economic losses (Song et al., 2014; Zhou et al., 2020). Previous studies have indicated that the C1qDC protein plays a crucial biological role in the innate immune defense of organisms against bacterial infections (Xiong et al., 2021). To understand the immune defense of *C. idella* in response to bacterial infection, a novel C1qDC gene (*Ci*C1ql2) was cloned from *C. idella*. In addition, the expression pattern, binding activity, and role of *Ci*C1ql2 in the intestinal defense against bacterial infections *via* classical complement pathway activation were also investigated. In-depth research on the function of C1qDCs in the immune system of *C. idella*, as well as the underlying mechanisms, will provide a theoretical foundation for the development of related strategies to further enhance immune responses.

2. Materials and methods

2.1. Animals, immune challenge and sample collection

C. idella, weighing approximately 30 ± 1.0 g, were obtained from the Hunan Institute of Aquatic Science in Hunan Province, China. The fish were maintained in the laboratory with a recirculating water system at 25 °C for 2 weeks. For the tissue-specific expression pattern analysis, the heart, liver, spleen, kidney, intestine, muscle, brain and gills were harvested from three healthy fish. The collected samples were promptly homogenized in RNAiso Plus (Accurate Biology, China), rapidly frozen in liquid nitrogen, and subsequently stored at − 80 ℃ before use.

For the *A. hydrophila* and *A. veronii* challenge experiments, the fish were divided into three groups of 20 individuals each and placed in tanks with 50 L of water. In the experimental groups, the fish were immersed in water containing *A. hydrophila* (5×10^7 CFU/ml), *A. veronii* (2×10^7 CFU/ml) or PBS. The intestines were collected from three fish in each group at different time points (3, 6, 9, 12, and 24 h) after challenge and stored in liquid nitrogen until further use. To investigate the impacts of LPS and PGN challenge on *C. idella*, three groups of 20 fish each were intraperitoneally injected with 10 mg/kg LPS (Beyotime, China) or 10 mg/kg PGN (Beyotime, China). The control group was injected with the same volume of sterile PBS (pH 7.4). At 12, 24, 48, and 72 h post injection (p.i.), intestines were collected from 3 fish in each challenge group for subsequent gene expression analysis. All the experiments were conducted in accordance with the national regulations on the use of laboratory animals of China and were approved by the Animal Ethics Committee of Changsha University.

2.2. cDNA cloning, identification of CiC1ql2 and sequence analysis

The *Ci*C1ql2-F1 and *Ci*C1ql2-R1 (Table 1) primers were designed in accordance with the sequence information of *Ma*C1ql2 (XM_048162858.1), which was acquired from the NCBI database (https://www.ncbi.nlm.nih.gov/). The PCR template was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Japan). The optimal PCR mixture was composed of 25 μl of premixed Taq (Ex Taq HS Version, Takara), 2 μl of each primer (10 μM), 2 μl of template, and 19 μl of ddH$_2$O. The PCR program consisted of an initial denaturation at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 40 °C for 30 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The obtained PCR products were analyzed *via* 1.5% agarose gel electrophoresis and purified *via* a DNA Gel Extraction Kit (Accurate Biology, China). The PCR products were subsequently inserted into the pMD18-T vector (Takara, Japan) and sequenced by Biosune (Shanghai, China).

A sequence homology search of the putative *Ci*C1ql2 sequence was carried out using the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the conserved domain features were predicted by the SMART program (http://smart.emblheidelberg.de/). Multiple sequence alignments were performed using ClustalX1.83, and the phylogenetic tree was constructed using the boot-strapped neighbor joining method of MEGA version 5.0.

2.3. CiC1ql2 protein expression and purification

The cDNA sequence that encodes the mature protein was amplified with the specific primers *Ci*C1ql2-F2 and *Ci*C1ql2-R2. The *Nde* I and *Xho* I restriction sites were added to the 5′ ends of *Ci*C1ql2-F2 and *Ci*C1ql2-R2, after which the PCR products were purified and inserted into the pMD18-T vector. The pMD18-T-*Ci*C1ql2 and plasmid pET32a were digested with *Nde* I and *Xho* I and then ligated using solution I (TaKaRa, Japan). The recombinant plasmid pET32a-*Ci*C1ql2 was transformed into *Escherichia coli* BL21 (DE3) (Sangon Biotech, China), which was subsequently cultured in LB supplemented with kanamycin at 37 °C. When the culture medium reached an O.D. 600 of 0.6, isopropyl-β-D- thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM, and the mixture was incubated at 37 °C overnight. The cells were collected by centrifugation at 4000 rpm for 10 min at 4 °C, after which the precipitate was resuspended in 1 × PBS. Protein purification was performed using Ni-NTA (Sangon Biotech, China) according to the manufacturer's protocol. The purified protein was dialyzed against 1 × PBS, enriched with 20,000 PEG, and stored at − 80 °C after being filtered through a 0.45 μm membrane (Sangon Biotech, China). Eventually, the protein concentration was measured by a protein concentration quantitative kit (Sangon Biotech, China).

2.4. Western blotting analysis

The purified *Ci*C1ql2 protein was subjected to SDS—PAGE. After electrophoresis, the proteins were transferred to PVDF membranes (Sangon Biotech, China). The PVDF membranes were washed with 1 × TBST three times at each step and then blocked with 5% SMP (Beyotime, China) for 2 h. The primary antibody used was a monoclonal mouse anti-

Table 1
Sequences of designed primers used in this study.

Primer	Sequence (5′ to 3′)	Comment
*Ci*C1ql2-F1	TCAGGTAAAGATGTCCT	CDS Cloning
*Ci*C1ql2-R1	AGACTCAGTAATGCCAATAA	
*Ci*C1ql2-F2	AATTTTGTTTAACTTTAAGAAGGAGATATACATATGCACCATCACCATCACCATGAAGAAAAGGG	Protein expression
*Ci*C1ql2-R2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTACAGACTATACAGCAGATGACCCACAA	
*Ci*C1ql2-F	CTGAAGAACAGACTGGAGGT	Real-Time PCR
*Ci*C1ql2-R	AAACACATCTTGGTAAATCA	
β-actin-F	GCTATGTGGCTCTTGACTTCG	Real-Time PCR
β-actin-R	GGGCACCTGAACCTCTCATT	
C3-F	GTCTCATTATGGCGGTTCTGG	Real-Time PCR
C3-R	TTCACCTGCGTGCGATACTCT	
C5-F	AGCGGGCACTCATACACTCAC	Real-Time PCR
C5-R	ATTCCCTCTGGCACCACTTTC	
C7-F	CCAGCACCTGAGTCTGTCCGA	Real-Time PCR
C7-R	GGCAGATGCTCATTCCGTTC	
CR1-F	GGAAACCTGAACCACCAAAGTG	Real-Time PCR
CR1-R	GTCCCATCCTCTGAACAACGAA	
TNF-α-F	CATCCATTTAACAGGTGCATAC	Real-Time PCR
TNF-α-R	GCAGCAGATGTGGAAAGAGAC	
IL-1β-F	GCCAAGTAGCCGAATCACAGA	Real-Time PCR
IL-1β-R	AGAAGCCCAAGATATGCAGGA	
IL-8-F	CATGTCTGACCATTACTGAAGC	Real-Time PCR
IL-8-R	GTTTCCTTCAGGGTGGCAATG	
siRNA-NC-F	UUCUCCGAACGUGUCACGUTT	NC siRNA
siRNA-NC-R	ACGUGACACGUUCGGAGAATT	
siRNA-1-F	CACUUGGACCCAUUGGUAATT	*Ci*C1ql2 siRNA
siRNA-1-R	UUACCAAUGGGUCCAAGUGTT	
siRNA-2-F	GGAAGAGCUUACAACCCAATT	*Ci*C1ql2 siRNA
siRNA-2-R	UUGGGUUGUAAGCUCUUCCTT	
siRNA-3-F	GACACAGGAUCUAAUUCAATT	*Ci*C1ql2 siRNA
siRNA-3-R	UUGAAUUAGAUCCUGUGUCTT	

His tag antibody (1:5000, the optimal dilution determined previously) (Sangon Biotech, China), after which the membranes were incubated for 2 h. The secondary antibody used was a goat anti-mouse antibody (1:8000) (Sangon Biotech, China), which was incubated with the membranes for 2 h. The antibody-bound protein was visualized by TMB (Sangon Biotech, China).

To explore the function of the recombinant *Ci*C1ql2 protein (r*Ci*C1ql2) in bacterial agglutination, a r*Ci*C1ql2 and bacteria binding experiment was performed following the methods described by Lee and Söderhäll (Lee and Söderhäll, 2001). The gram-negative bacteria used in the experiment included *A. hydrophila* and *A. veronii*, while the gram-positive bacterium was *B. subtilis*. The bacteria were seeded in LB liquid culture medium and incubated overnight at 200 rpm and 30 °C. The bacteria were diluted to a concentration of 1×10^8 CFU/ml with PBS. Then, 1 ml of the bacteria was incubated with 100 μg of r*Ci*C1ql2. The precipitate was collected by centrifugation. Finally, western blotting was performed to complete the experiment.

2.5. Cell culture and rCiC1ql2 treatment experiment

The *C. idella* intestinal cells from our laboratory (Zhou et al., 2022) were cultured in DMEM/F12 (Gibco, USA) supplemented with 10% FBS (Gibco, USA), penicillin (100 U/ml) (Gibco, USA), and streptomycin (100 mg/ml) (Gibco, USA) and maintained at 28 °C in CO_2 (5%) a humidified incubator.

For the *in vitro* experiments, after cell subculture, the passaged *C. idella* intestinal cells were seeded into 6-well plates and randomly divided into four groups (three wells per group). The four groups included the blank control group, r*Ci*C1ql2 treatment group (10 μg/ml), LPS treatment group (10 μg/ml), and r*Ci*C1ql2 and LPS combined treatment (10 μg/ml+10 μg/ml). After passaging, the cells had adhered well. The original culture medium was removed, and then, the cells were gently washed with 2 ml PBS. Complete cell medium containing different concentrations of r*Ci*C1ql2 and LPS was added to each group of cells. After culture for 12, 24, 48 h, the cell culture medium was removed, and then the cells were washed with 1 ml PBS three times. The cells were collected with RNAiso Plus (Accurate Biology, China), frozen in liquid nitrogen, and stored at − 80 °C for use in subsequent experiments.

2.6. RNA interference

Three pairs of *Ci*C1ql2-specific siRNAs and scrambled siRNA (negative control; NC siRNA) are listed in Table 1, and these molecules were chemically synthesized by Shanghai GeneBio. *C. idella* (weighing approximately 4 ± 1.0 g) were randomly divided into four groups (three fishes per group) that were housed in the indoor circulating aquaculture system. Different types of siRNA (1 μg/g) (Ge et al., 2020) were injected into the abdomen of the fish, and intestinal tissues were sampled 48 h after injection; siRNAs with the best gene knockdown effects were identified by qRT—PCR. The formal experiment included the NC group, siRNA-NC+LPS group, and siRNA-1 +LPS group, and the treatments were administered according to body weight. Three groups of 3 fish per group were injected with the same volume (100 μl) of siRNA-NC, siRNA-NC, or siRNA-1. After 48 h, the NC group was injected with 100 μl siRNA-NC, and the other two groups were injected with 100 μl LPS. *C. idella* individuals were anesthetized with 20 mg/l MS-222 (Sigma—Aldrich), and then after 24 h, intestinal tissues were collected for subsequent qRT—PCR analysis.

2.7. RNA extraction and quantitative real-time PCR analysis

Total RNAs were extracted by using RNAiso Plus (Accurate Biology, China) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were assessed by spectrophotometer (Thermo, USA), and then, the quality of the RNA was analyzed by 1.0% agarose gel. Afterward, cDNA was synthesized from 1.0 μg of each RNA sample using a Evo M - MLV Mix Kit with gDNA Clean for qPCR (Accurate Biology, China).

After reverse transcription of the RNA, quantitative real-time PCR (qRT—PCR) was performed with a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, USA). The qRT—PCR mixture consisted of 8

μl of 2 ×SYBR Green *Pro Taq* HS Premix II (High Rox Plus) (Accurate Biology, China), 1 μl of cDNA, 5.72 μl of RNase-free water, and 0.64 μl of each specific primer. Each assay was carried out in triplicate with the following cycling procedure: 95 °C for 5 min for activation, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. The specific primers for the genes that were examined by qRT—PCR are listed in Supplemental Table 1. The expression levels of all the target genes were analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001), with β-actin serving as an internal reference.

2.8. Statistical analysis

All the data were shown as the mean ± S.E (n = 3), and the data were analyzed by one-way ANOVA with the SPSS 26 (Chicago, IL, USA) statistical software package. Significant differences among the groups were confirmed using one-way analysis of variance with Tukey's multiple range tests. P values less than 0.05 and 0.01 were considered statistically significant and extremely significant, respectively.

A

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1              M  S  C  T  I  L  Y  P  L  L  L  L  L  F  S  C  A
1 ttcaggtaaagATGTCCTGCACCATTCTGTATCCGCTGCTGCTTCTCCTGTTTAGCTGTG
18  C  L  S  E  V  Q  Q  E  E  K  G  V  L  T  E  S  P  S  N  E
61 CCTGTCTGTCTGAGGTCCAGCAGGAAGAGAAGGGAGTACTGACTGAGAGTCCTTCGAATG
38  E  E  S  G  A  V  L  T  S  G  E  F  Q  Q  R  F  L  T  G  I
121 AAGAGGAAAGCGGTGCTGTTTGACTTCTGGTGAGTTTCAGCAACGCTTTCTCACAGGGA
58  Y  S  E  L  A  E  L  R  S  T  V  R  S  L  K  N  R  L  E  V
181 TCTACTCTGAGCTGGCAGAGCTGAGATCAACTGTGAGATCTCTGAAGAACAGACTGGAGG
78  T  E  E  Q  I  R  K  K  E  Y  K  V  A  F  A  A  T  L  G  P
241 TCACTGAGGAGCAGATCAGGAAAAAAGAATATAAAGTGGCATTTGCCGCCACACTTGGAC
98  I  G  N  L  G  P  F  N  T  E  I  T  L  I  Y  Q  D  V  F  V
301 CCATTGGTAACCTTGGACCTTTTAACACTGAGATCACTCTGATTTACCAAGATGTGTTTG
118  N  E  G  R  A  Y  N  P  T  T  G  I  F  T  A  P  V  K  G  V
361 TGAATGAAGGAAGAGCTTACAACCCAACCACTGGTATCTTCACAGCACCTGTTAAAGGTG
138  Y  F  F  I  I  T  G  H  N  R  S  S  R  S  M  G  L  R  L  F
421 TCTATTTCTTCATTATCACTGGACATAACCGCTCATCTAGAAGTATGGGTCTAAGACTCT
158  K  N  G  Q  Q  M  I  T  I  Y  N  H  A  L  G  D  R  Y  D  T
481 TTAAAAATGGACAACAGATGATAACAATTTACAATCATGCTCTAGGTGACCGCTATGACA
178  G  S  N  S  I  S  L  T  L  E  E  G  D  H  V  Y  V  R  L  R
541 CAGGATCTAATTCAATCTCTTTGACTCTAGAGGAAGGAGATCATGTCTATGTGCGTCTCC
198  E  N  T  W  V  F  D  N  V  N  D  H  T  S  F  V  G  H  L  L
601 GGGAAAATACATGGGTCTTTGATAATGTAAATGACCACACTTCATTTGTTGGCCATTTAC
218  Y  S  L  *
661 TTTATTCTCTTTGAtggtcttcaatgaacatcatcttatgaggctaaagtcttaatgttt
721 gttgagtcatatttcatgttatttggtagttctattaaaatgaactgcttggaaaattta
781 aactttttaaagaaacttaatttgtgcttttagctttcatcgtctcttacaatagactt
841 acagtaattattggcattactgagtc
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B

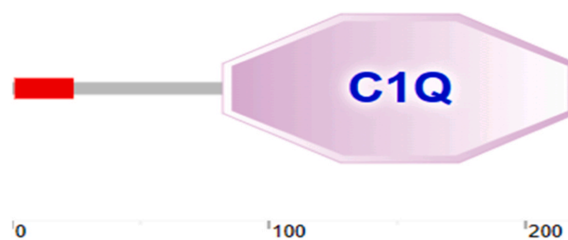

Fig. 1. The cDNA sequence of *Ci*C1ql2. (A) The signal peptide is highlighted. The C1q domain is indicated in gray. (B) The domain organization was predicted by SMART. The red region is the signal peptide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

3. Results

3.1. Molecular characterization of CiC1ql2

The cDNA sequence of *Ci*C1ql2 was cloned from *Ctenopharyngodon idella* and obtained from NCBI GenBank with the accession number of OR413255. The *Ci*C1ql2 sequence consisted of a 660-bp ORF encoding 220 amino acids (Fig. 1A), and it had a predicted molecular weight of 24.8 kDa and theoretical isoelectric point of 5.42. Similar to other C1qls, *Ci*C1ql2 was composed of a C1q domain and a signal peptide according to SMART prediction (Fig. 1B).

The multiple C1ql2 amino acid sequence alignments showed that the *Ci*C1ql2 protein exhibits low sequence identity with the C1ql sequences from other species except for the critical conserved C1q domain and signal peptide (Fig. 2). To investigate the evolutionary relationships of C1qls, a phylogenetic tree was constructed using the C1ql sequences of fourteen representative vertebrate species. *Ci*C1ql2 shared 88.29% amino acid sequence identity with a homolog from *Megalobrama amblycephala* (XP_048018815.1) and 86.04% amino acid sequence identity with a homolog from *Anabarilius grahami* (ROL44151.1). Phylogenetic analysis revealed that *Ci*C1ql2 first clustered with C1ql2 from *M. amblycephala* and then clustered with other teleost homologs, revealing the closest relationship of C1ql2 proteins between *C. idella* and *M. amblycephala*. The C1ql proteins from other vertebrates, such as *Homo sapiens*, *Mus musculus*, *Rhinolophus ferrumequinum* and so on, consecutively formed sister groups of the group formed by the teleost C1ql protein (Fig. 3). These observations suggested that C1ql proteins might be conserved from fish to mammals throughout the evolution of vertebrates.

3.2. Tissue expression analysis of CiC1ql2

qRT—PCR was used to measure the expression of *Ci*C1ql2 in various tissues of *C. idella*, and the results are shown in Fig. 4. *Ci*C1ql2 was expressed in all the analyzed tissues, namely, heart, liver, spleen, kidney, intestine, muscle, brain and gill, with the highest expression in the intestine, followed by liver, and minimal expression in the brain. The constitutive expression of *Ci*C1ql2 revealed the involvement of *Ci*C1ql2 in multiple physiological processes of *C. idella*.

3.3. Analysis of CiC1ql2 expression in response to bacterial pathogen infection and PAMP exposure

The expression of *Ci*C1ql2 was examined in fish in response to *A. hydrophila* and *A. veronii* infection (Fig. 5 A). In the *A. hydrophila* treatment group (Fig. 5 A), the expression of *Ci*C1ql2 was remained high after infection with *A. hydrophila*. However, in control group, the expression of *Ci*C1ql2 was comparatively lower than that in the *A. hydrophila* treatment group. The expression level of *Ci*C1ql2 in the *A. hydrophila* treatment group significantly increased from 3 h to 24 h p.i., with increases of approximately 20.5-fold, 41.2-fold, 3.5-fold, 404.0-fold and 103.3-fold compared to the control group, respectively. In the *A. veronii* treatment group, the mRNA expression levels of *Ci*C1ql2 increased from 3 h p.i.(2.7-fold), and peaked at 6 h p.i.(143.7-fold), and then decreased, showing a trend of initial increase followed by a decrease. Additionally, at 24 h p.i.(1.2-fold), the expression level of *Ci*C1ql2 was almost consistent with that of the control group.

In the PGN injection group (Fig. 5B), the relative expression of *Ci*C1ql2 first decreased at 24 h postinjection and then increased at 48 h postinjection, and the control group showed similar trends. Moreover, the expression levels of *Ci*C1ql2 in the PGN injection group were consistently higher than those in the control group. However, the expression of *Ci*C1ql2 in the LPS injection group was significantly upregulated compared with that in the control group, with peak increase of approximately 2.8-fold. These results suggested that *Ci*C1ql2 might be involved in immune defense against bacterial challenges.

3.4. Analysis of bacterial aggregation activity of rCiC1ql2

E. coli BL21 (DE3) cells harboring the recombinant expression construct pET32a-*Ci*C1ql2 successfully expressed the His-*Ci*C1ql2 fusion protein after incubation with 0.5 mM IPTG (Fig. 6A). The subsequent purification steps utilized the supernatant that was obtained after homogenization and centrifugation. The His-*Ci*C1ql2 fusion protein was effectively purified using Ni-NTA, as demonstrated by SDS—PAGE (Fig. 6B). To further evaluate the possible immune function of *Ci*C1ql2 in fish, an *in vitro* bacterial aggregation experiment was performed. As shown in Fig. 6C, three separate bacterial suspensions, including the gram-negative bacteria *A. hydrophila* and *A. veronii* and the gram-positive bacteria *B. subtilis,* were used as negative controls respectively, and the recombinant protein r*Ci*C1ql2 was used as the positive control. The results revealed that all three types of bacteria could significantly bind to the r*Ci*C1ql2 protein, indicating that the r*Ci*C1ql2

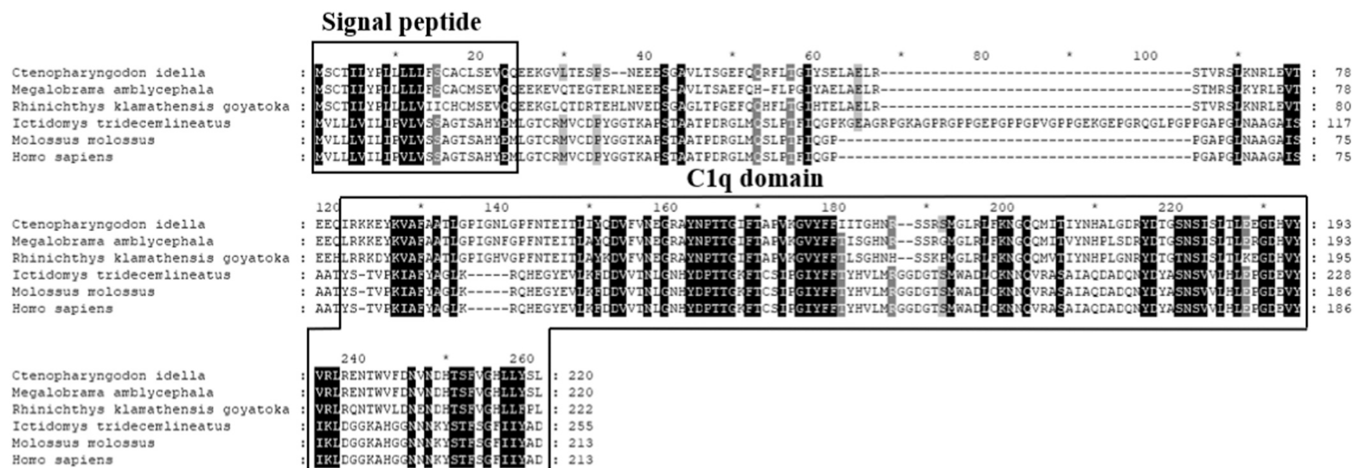

Fig. 2. Alignment of the predicted amino acid sequences of *Ci*C1ql2 homologs. The percentage in the bracket following each species name indicates the overall sequence identity between *Ci*C1ql2 and the protein of the particular species. The Gene Bank accession numbers of the aligned sequences are as follows: *Megalobrama amblycephala* (XP_048018815.1), *Rhinichthys klamathensis goyatoka* (XP_056097124.1), *Molossus molossus* (KAF6499253.1), *Ictidomys tridecemlineatus* (KAG3257554.1), and *Homo sapiens* C1ql3 (KAI4075349.1).

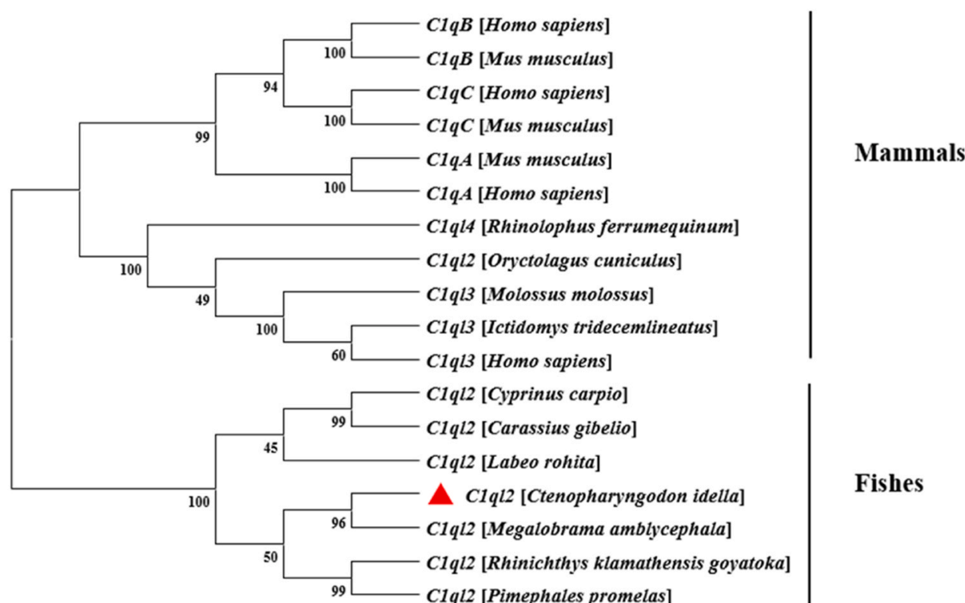

Fig. 3. Phylogenetic analysis of *Ci*C1qlp2 and other C1qDC proteins. The phylogenetic tree was constructed with MEGA 11 software (http://www.megasoftware.net/) using the neighbor-joining method. CiC1qlp2 is indicated by a triangle. Numbers next to the internal branches indicate bootstrap values based on 1000 replications. The accession numbers of the analyzed sequences are as follows: *Megalobrama amblycephala* C1ql2 (XP_048018815.1), *Ctenopharyngodon idella* C1ql2 (OR413255), *Labeo rohita* C1ql2 (KAI2646595.1), *Cyprinus carpio* C1ql2 (XP_042623544.1), *Carassius gibelio* C1ql2 (XP_052386651.1), *Rhinichthys klamathensis goyatoka* C1ql2 (XP_056097124.1), *Pimephales promelas* C1ql2 (XP_039510469.1), *Triplophysa rosa* C1ql2 (XP_057190421.1), *Mus musculus* C1qA (NP_031598.2) , *Homo sapiens* C1qA (NP_057075.1), *Mus musculus* C1qB (NP_033907.1), *Homo sapiens* C1qB (NP_000482.3), *Mus musculus* C1qC (NP_031600.2), *Homo sapiens* C1qC (NP_758957.2), *Molossus molossus* C1ql3 (KAF6499253.1), *Ictidomys tridecemlineatus* C1ql3 (KAG3257554.1), *Homo sapiens* C1ql3 (KAI4075349.1), *Rhinolophus ferrumequinum* C1ql4 (KAF6338948.1), and *Oryctolagus cuniculus* C1ql2 (XP_051705825.1).

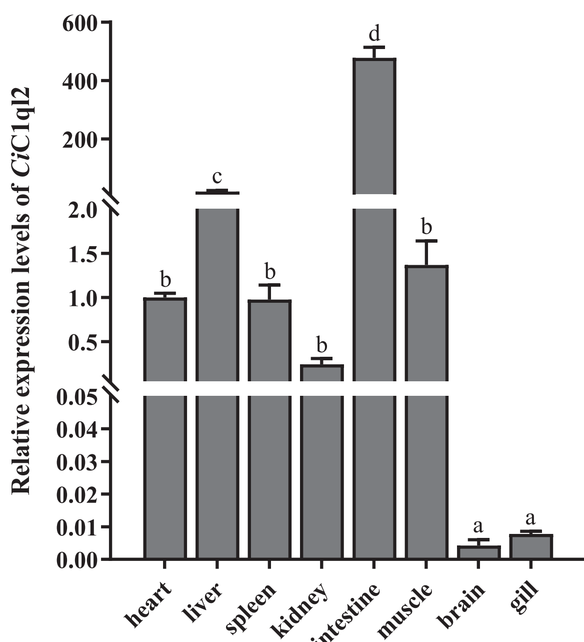

Fig. 4. The gene expression levels of *Ci*C1ql2 in different tissues of *C. idella*. All the data are expressed as the mean ± SE (n = 3). Significant differences are indicated with different letters (P < 0.05).

protein possessed bacterial agglutination abilities, which suggested that the r*Ci*C1ql2 protein might be involved in innate immune responses in *C. idella*.

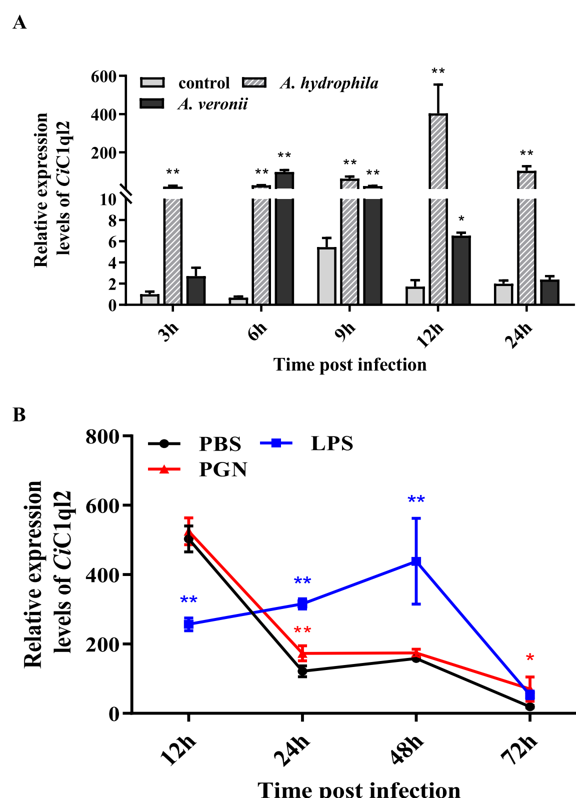

Fig. 5. (A) Relative expression of *Ci*C1ql2 in the intestine after *A. hydrophila* and *A. veronii* infection. (B) Relative expression of *Ci*C1ql2 in the intestine after LPS and PGN injection. All the data are expressed as the mean ± SE (n = 3). Significant differences between the infected and control groups at the same time point are indicated with * (P < 0.05) or * *(P < 0.01).

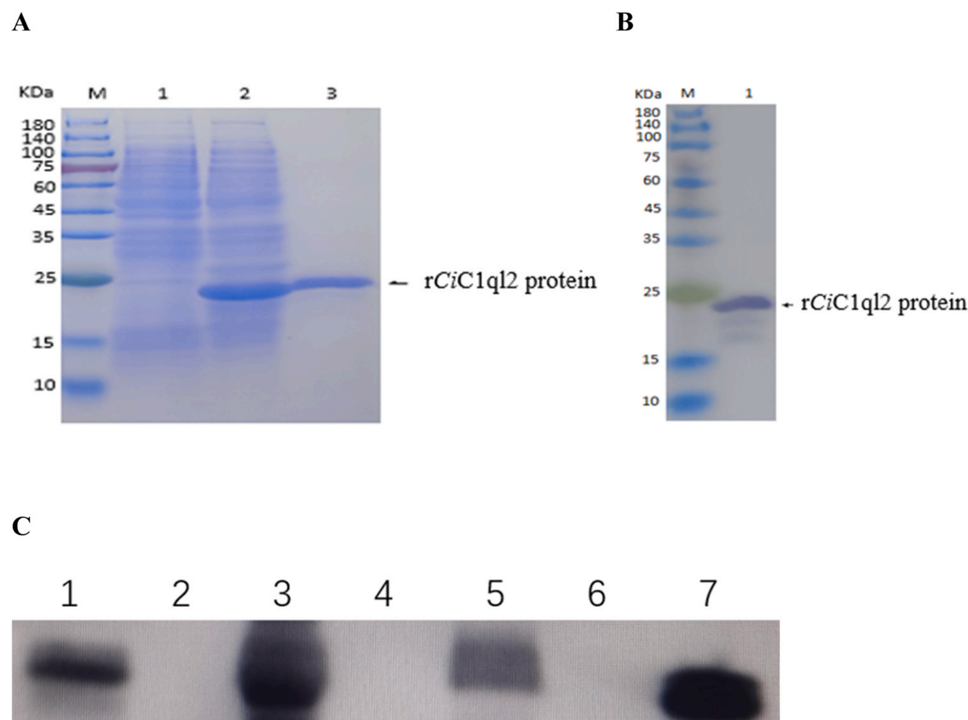

Fig. 6. SDS—PAGE (A), western blotting (B) analysis of the recombinant r*Ci*C1ql2 protein and western blotting (C) analysis of the r*Ci*C1ql2 protein binding to the gram-negative bacteria *A. hydrophila*, *A. veronii* and the gram-positive bacteria *B. subtilis*.(A) M: Protein Marker; Lane1: Total protein before induction; Lane 2: Total protein after induction; Lane 3: r*Ci*C1ql2 protein.(B) M: Prestained Marker; Lane1: r*Ci*C1ql2 protein. (C) Lane 1: r*Ci*C1ql2 protein; Lane 2: *A. hydrophila*; Lane 3: r*Ci*C1ql2 protein bound to *A. hydrophila*; Lane 4: *A. veronii*; Lane 5: r*Ci*C1ql2 protein bound to *A. veronii*; Lane 6: *B. subtilis*; Lane 7: r*Ci*C1ql2 protein bound to *B. subtilis*.

3.5. Effects of the rCiC1ql2 protein on intestinal immune responses of C. idella

To investigate whether the r*Ci*C1ql2 protein is important for protection against LPS, the mRNA expression of selected classic complement genes and inflammatory cytokine genes in *C. idella* intestinal cells were measured at 12, 24, 48 h p.i. (Fig. 7). In the r*Ci*C1ql2 treatment group, the expression of CR1 was upregulated from 12 h to 48 h p.i., peaking at the 24 h p.i., compared with the control group. The expression of C5 and C7 in the r*Ci*C1ql2 treatment group were also upregulated compared to the control group. Additionally, the mRNA expression of TNF-α, IL-1β and IL-8 were significantly upregulated at 12 h p.i., 24 h and 48 h p.i., and 12 h, 24 h, 48 h p.i., respectively than the control group. After challenge with LPS, the expression of IL-8 were gradually significantly upregulated and peaked after 48 h p.i. The expression of TNF-α and IL-1β were significantly upregulated at 12 h p.i. and 12 h, 24 h, 48 h p.i., after stimulations with LPS compared to the control group. In the LPS treated group, the mRNA expression of CR1, C3, C5 and C7 were significantly upregulated at 24 h and 48 h p.i. compared to the control group. In the r*Ci*C1ql2 + LPS treated group, the expressions levels of TNF-α, IL-1β and IL-8 were significantly upregulated at 24 h p.i., 12 h and 48 h p.i., and 12 h and 48 h p.i. in intestine compared with the LPS treatment group. In addition, the mRNA levels of classical complement pathway related genes were upregulated in the r*Ci*C1ql2 + LPS treated group compared to the LPS treated group at 48 h p.i. It was preliminarily revealed that r*Ci*C1ql2 may affect the expression of genes in the classical complement pathway, and subsequently affected intestinal inflammation in *C. idella*.

3.6. Effects of CiC1ql2 knockdown on intestinal immune responses in C. idella

To obtain evidence to support the activation of the classic complement pathway by *Ci*C1ql2 *in vivo*, we performed further experiments to analyze the effect of *Ci*C1ql2 in activating the classic complement pathway in *C. idella*. As shown in Fig. 8A, the expression of *Ci*C1ql2 was significantly knocked down 48 h after transfection with siRNA-1, siRNA-2, and siRNA-3 compared with PBS treatment, and the sequence with the greatest knockdown efficiency (8.2-fold) was siRNA-1. Subsequent experiments were conducted using siRNA-1 combined with LPS treatment. In the LPS treatment group, the expression of CR1 and classic complement genes (C3, C5, and C7), inflammatory cytokines (TNF-α, IL-1β and IL-8) were generally increased compared to the NC group, indicating that LPS has induced intestinal inflammation. The expression of *Ci*C1ql2 was knocked down in siRNA-1 + LPS-injected *C. idella*, and the mRNA levels of *Ci*C1ql2 was 0.1-fold ($p < 0.05$) compared with that in the NC + LPS-injected group, and it was lower than that in the NC group (Fig. 8B). The mRNA expression levels of classic complement genes (C3, C5, and C7) in the *Ci*C1ql2-knockdown *C. idella* were significantly decreased at 24 h ($p < 0.05$) after LPS stimulation, compared with that in the NC + LPS-injected group, respectively, suggesting that *Ci*C1ql2 might be involved in the classical complement signaling pathway. In addition, the transcript levels of IL-1β and IL-8 in the siRNA-1 + LPS group were significantly decreased compared to those in the NC + LPS group. Although the expression of TNF-α was also decreased in the siRNA-1 + LPS group compared with the NC + LPS group, the difference was not significant. These results suggested that *Ci*C1ql2 might participate in the LPS-induced immune response in the intestines of *C. idella*.

4. Discussion

C1qDC proteins that contain the C1q domain form a family of signaling molecules in the complement pathway, and these proteins have been identified in a variety of animals (Bally et al., 2009; Huang et al., 2016; Liang et al., 2022; Mei and Gui, 2008). C1ql was an

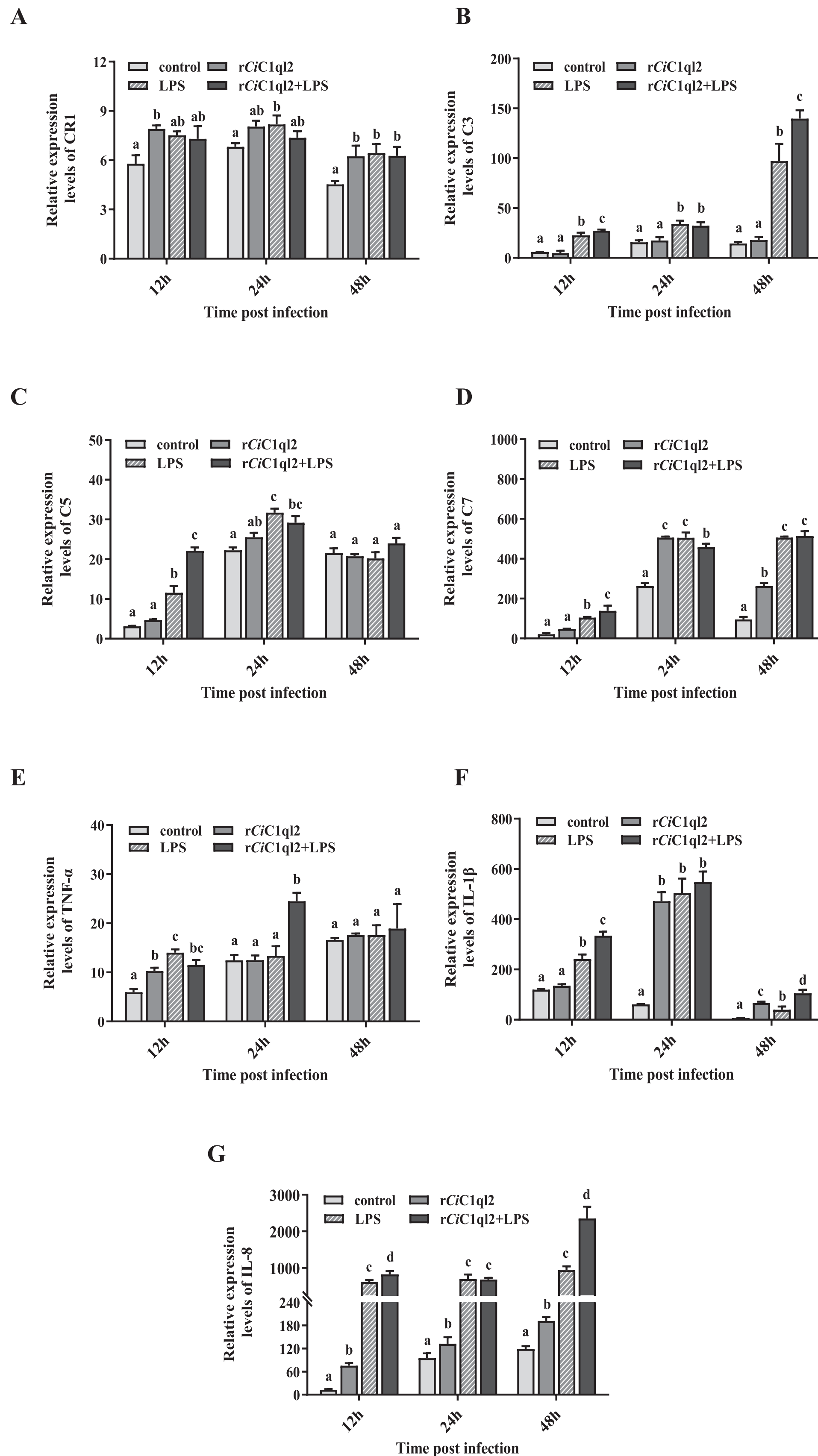

Fig. 7. Relative expression of CR1, C3, C5, C7, TNF-α, IL-1β, and IL-8 in the intestinal cells after r*Ci*C1ql2 and LPS treatment. All the data are expressed as the mean ± SE (n = 3). Bars bearing the different letters are significantly different according to one-way 1uanalysis of variance (ANOVA) ($P < 0.05$).

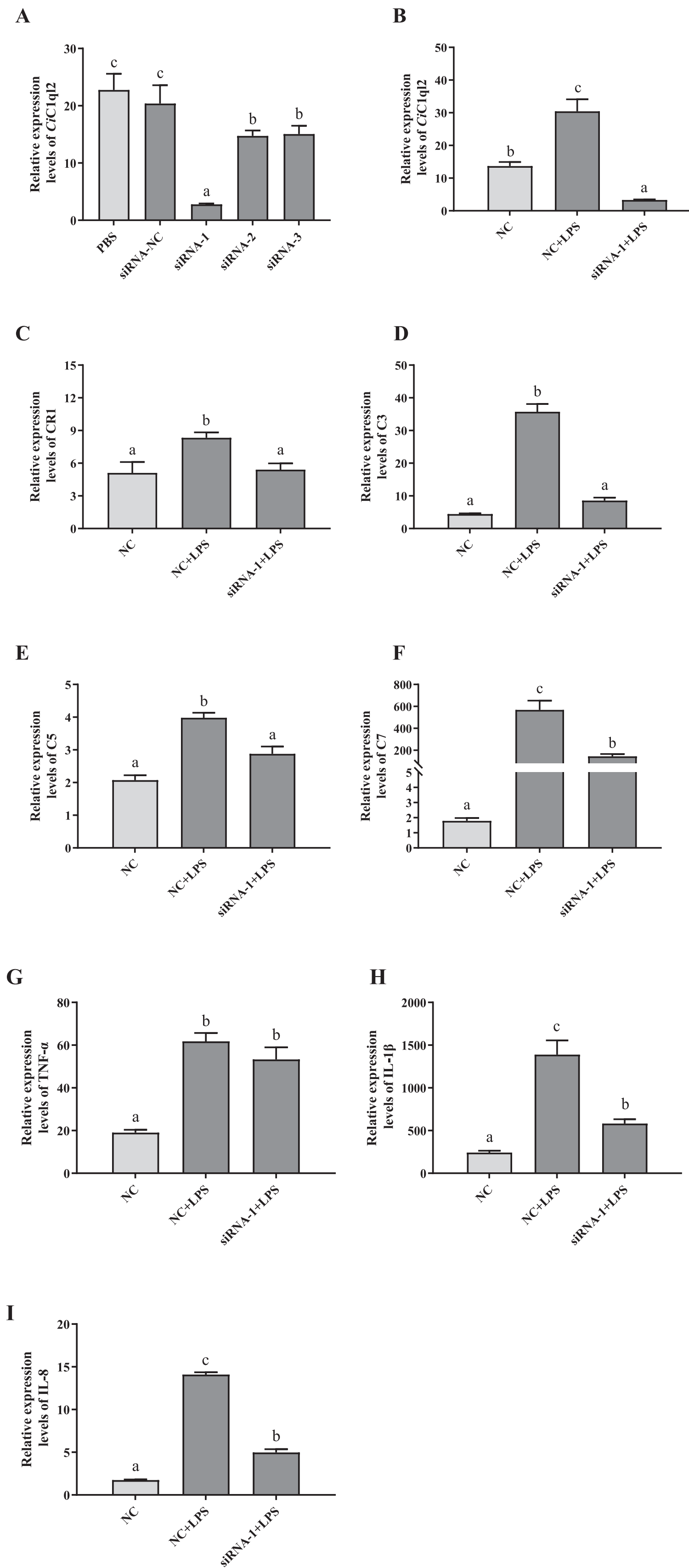

Fig. 8. Relative expression of CR1, C3, C5, C7, TNF-α, IL-1β, and IL-8 after RNA interference of *Ci*C1ql2 *in vivo*. All the data are expressed as the mean ± SE (n = 3). Bars bearing the different letters are significantly different according to one-way analysis of variance (ANOVA) (P < 0.05).

important member of the C1qDC protein family and was essential for innate immunity in mammals, but its function has been poorly studied in fish, and only one C1qDC family member has been reported in zebrafish (Mei et al., 2008). In this article, a novel C1qDC protein called *Ci*C1ql2 was identified in *C. idella*. In addition to the C1q domain and coiled-coil domain, the deduced *Ci*C1ql2 protein also contained a signal peptide at its 5′ end according to SMART analysis; this structure was consistent with the protein domain of *Cc*C1qDC from Yellow River carp (Zhu et al., 2021). Therefore, *Ci*C1ql2 was also presumed to function as a secreted protein. The deduced amino acid sequence of *Ci*C1ql2 shared a relatively high similarity with those of C1ql proteins from fish species, such as *M. amblycephala*, *C. carpio* and so on. In the phylogenetic tree, *Ci*C1ql2 was first clustered with *Ma*C1ql2 from *M. amblycephala* in the fishes C1ql clade. This result suggested that *Ci*C1ql2 was a C1qDC family member and that it might perform similar functions. *Cs*sghC1q was shown to possess antibacterial and antiviral activities by recognizing binding ligands and inducing phagocytosis, thus participating in the immune response of *Cynoglossus semilaevis* (Chen et al., 2017; Zeng et al., 2015). T.M. (Carland et al., 2012) reported that the expressions of sghC1q08 and sghC1q09 in zebrafish was significantly upregulated 24 h after infection with the bacterial pathogen *Streptococcus iniae*, revealing that sghC1q participated in innate immune responses. It could be indicated that *Ci*C1ql2, which is one member of the fish C1qDC family, might perform similar functions and participate in immune defense processes of *C. idella*.

The intestine is not only a vital organ for digestion and absorption in animals but also an important immune organ for the resistance of pathogens (Cui et al., 2021; Taylor et al., 2021; Vargas-Albores et al., 2021). This conclusion is because while substances are absorbed and metabolized in the intestine, the intestine is also a site of direct contact with pathogens such as bacteria, fungi and viruses, which is particularly crucial for fish (Dawood, 2021; Palomino Ramos et al., 2022; Zhang et al., 2022). In the present study, the *Ci*C1ql2 was widely distributed in all the investigated tissues, with the highest expression in intestinal tissues. The relatively high expression of *Ci*C1ql2 in intestinal tissues suggested that *Ci*C1ql2 was an important component of the intestinal immune system. Our results were consistent with those of (Pei et al., 2016) who reported the tissue distribution of L-C1qDC-1 in *Lethenteron camtschaticum*. After stimulation with *A. hydrophila* and *A. veronii*, *Ci*C1ql2 transcription was significantly upregulated and then slightly decreased but remained higher than that in the control group; this trend was similar to that of the *Cg*C1qDC-1 and *Pf*-ghC1q mRNA levels after bacterial infection (Jiang et al., 2015; Wang et al., 2022). The transcription level of *Ci*C1ql2 was significantly upregulated after bacterial stimulation, revealing that *Ci*C1ql2 could be rapidly upregulated by *A. hydrophila* and *A. veronii* effectively, thereby triggering the production of immune molecules to protect against pathogen invasion (Li, J. et al., 2023). LPS and PGN, which are main components of the cell walls of gram-negative bacteria and gram-positive bacteria, respectively, promoted inflammatory responses in animals (Moreillon and Majcherczyk, 2003). After *C. idella* were injected with LPS and PGN, the relative expression level of *Ci*C1ql2 was significantly upregulated. Based on previous studies, after injection with bacteria and PAMPs, the expression of *Cg*C1qDC-5, *Cf*C1qDC and *Hc*C1qDC6 was significantly increased (Huang et al., 2017; Lv et al., 2019; Zhang et al., 2008). *Is*C1ql3 was significantly induced in the intestine of several ticks after feeding on *Borrelia burgdorferi*-infected mice (Tang et al., 2022). According to these studies, it is speculated that C1ql2 could participate in the intestinal immune response against bacterial challenges in *C. idella*.

The C1q subunit, which is a constituent of complement 1 (C1), is also present in C1qDC, which possessed the capacity to recognize pathogens and initiate the classical complement pathway. This process led to the engulfment and removal of exogenous pathogenic microorganisms through phagocytosis (Carland and Gerwick, 2010). The classical complement pathway is a crucial and specific mediator of various autoimmune and inflammatory diseases (Garcia et al., 2016), and it can trigger a series of chemotaxis and pro-inflammatory responses, such as inducing the expression of TNF-α and IL-8 (Yang et al., 2013). *In vivo* and *in vitro* experiments showed that the relative expression levels of C3, C5, C7, TNF-α, IL-1β, and IL-8 were significantly upregulated after stimulations with LPS. These results were similar to the findings of (Qiang et al., 2017), namely, that the C3 mRNA level significantly increased, which consequently increased the transcription of IL-1β and TNF-α, helping to activate the inflammatory response after *S. iniae* injection. The activation of C3 and C5, which are key components of the classical complement pathway, revealed that the classical complement pathway was involved in activation of the complement pathway and the occurrence of bacterium-induced immune inflammatory responses (Li et al., 2022; Wu et al., 2022). The transcripts of C3, C5, and C7, which are members of the classical complement pathway, were significantly increased after LPS treatment. Moreover, the expression levels of factors (C7) that form the membrane attack complex were upregulated, indicating that many membrane attack complexes were synthesized to induce macrophage phagocytosis and pathogen elimination (Lukácsi et al., 2020). IL-1β, IL-8 and TNF-α are pro-inflammatory cytokines that play crucial roles in the recruitment and activation of neutrophils during inflammation (Del Valle et al., 2020). In this study, TNF-α, IL-1β and IL-8 mRNA expression rapidly increased after LPS treatment. This indicated that after LPS treatment, the classical complement pathway might participate in regulating intestinal inflammation in *C. idella*. According to previous studies, human polymorphonuclear cells exhibited increased C3a and C5a expression after infection with *Cryptococcus neoformans* to promote IL-8 production (Vecchiarelli et al., 1998). *Propionibacterium acnes* induced inflammation by activating the classical and alternative complement pathways to stimulate the formation of C5a, inducing monocytes to secrete pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8 (Vowels et al., 1995). In the present study, the intestinal expression levels of complement genes (C3, C5, and C7) and proinflammatory factor genes (TNF-α, IL-1β and IL-8) in response to LPS challenge were significantly inhibited by *Ci*C1ql2 knockdown, suggested that *Ci*C1ql2 may play essential roles in the regulation of LPS-induced intestinal immune the response in *C. idella*. Similar results were reported in *Hyriopsis cumingii* (Huang et al., 2017) where the knockdown of *Hc*C1qDC6 inhibited the expression of two immune-related genes (tumor necrosis factor and whey acidic protein). These findings revealed that complex interactions occur among the complement system, humoral immunity, and cytokine regulation (Oosterhoff et al., 2022).

The ability of the C1q domain to recognize pathogens has been extensively studied (Ghai et al., 2007). In this study, the recombinant *Ci*C1ql2 protein was successfully purified, and its immune recognition ability was determined by western blotting. The results showed that r*Ci*C1ql2 could bind to *A. hydrophila, A. vieteri* and *B. subtilis* and recognize them, which suggested that the r*Ci*C1ql2 protein was involved in the innate immune responses of *C. idella*. Similar findings were observed in previous studies. *Po*C1qDC from *Paralichthys olivaceus* bound to bacteria and exhibited agglutination activity (M.-f Li et al., 2023; J. Li et al., 2023). Y. Huang et al. (Huang et al., 2017) found that the *Hc*C1qDC protein from *H. cumingii* was able to bind to gram-positive bacteria and gram-negative bacteria, and the rC1q3 protein could bind more strongly to gram-positive bacteria. These studies suggested that the innate immune recognition function of the C1ql protein is highly conserved in aquatic animals. To investigate the role of the r*Ci*C1ql2 protein in mediating bacterial-induced intestinal immune responses, we performed an experiment in which *C. idella* intestinal cells were treated with both the r*Ci*C1ql2 protein and LPS. The expression level of the C1q receptor (CR1) was increased in the r*Ci*C1ql2 protein treatment group, and interestingly, the expression levels of C7 was also significantly increased, which preliminarily suggested that the r*Ci*C1ql2 protein was involved in mediating the activation of the classical complement pathway. After combined treatment with the r*Ci*C1ql2 protein and LPS, the transcript levels of C3, C5, and C7 were higher. Previous studies also showed that the r*Po*C1qDC protein could bind to fish IgM, activating C3

to cause complement activation and defend against bacterial infections (M.-f Li et al., 2023; J. Li et al., 2023). In addition, the expression levels of TNF-α, IL-1β and IL-8 in *C. idella* intestinal cells were significantly increased in the r*Ci*C1ql2 protein-treated group, which was consistent with the results of (Chen et al., 2018) who discovered that the recombinant *On*C1qs protein upregulated the expression of IL-8 in adherent leukocytes *in vitro.* C1q, which is an inducer of pro-inflammatory activators, was produced in response to infection (van den Berg et al., 1998). Consistent with the results of this study, *Ci*C1ql2 also activated the expression of pro-inflammatory factors. After combined treatment with the r*Ci*C1ql2 protein and LPS, the expression of TNF-α, IL-1β and IL-8 was more significantly upregulated, which indicated that the r*Ci*C1ql2 protein promoted the induction of bacterium-induced intestinal inflammation in *C. idella*. Studies have shown that when the expression of *Hc*C1qDC5 was silenced during exposure to *Vibrio parahaemolyticus*, the mRNA expression level of *Hc*TNF was significantly reduced, suggesting that *Hc*C1qDC5 interacted with *Hc*TNF during bacterial exposure to promote antibacterial innate immunity (Huang et al., 2016). Therefore, considering these results, it was inferred that *Ci*C1ql2 could have a significantly positive effect on recognizing pathogens, inducing complement activation, and playing an important role in initiating innate immune responses.

In conclusion, a gene encoding a novel C1q domain-containing protein (*Ci*C1ql2) in *C. idella* was identified and characterized in this study. The *Ci*C1ql2 protein was found to possess conserved structural features with other species. The transcription levels of *Ci*C1ql2 were significantly upregulated in response to bacterial and PAMP exposure *in vivo* and *in vitro*. Additionally, the purified r*Ci*C1ql2 protein was capable of agglutinating bacteria and inducing the expressions of classic complement genes and inflammation cytokines. In summary, these results suggested that *Ci*C1ql2 might participate the immune responses induced by bacterial pathogens *via* the activation of the classical complement pathway. These findings will contribute to further understanding the role of C1ql2 proteins in the immune response to bacterial challenge in bony fishes.

Author statement

The work described has not been submitted elsewhere for publication, in whole or in part, and all authors have contributed to and approved the publication of the work.

CRediT authorship contribution statement

Fan Junde: Writing – review & editing. **Peng Ran:** Methodology, Investigation. **Qu Fufa:** Writing – original draft. **Yang Yalin:** Writing – review & editing. **Liu Zhen:** Writing – original draft, Supervision, Project administration. **Zhou Zhigang:** Writing – review & editing. **Zhang Xia:** Writing – original draft, Methodology, Investigation, Data curation. **Wu Ting:** Methodology, Investigation. **He Hao:** Methodology, Investigation. **Guo Meixing:** Methodology, Investigation. **Tang Jianzhou:** Data curation. **Li Jianzhong:** Writing – original draft. **Zhang Yuhan:** Methodology, Investigation. **Jin Kelan:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that the study was conducted without any commercial or financial affiliations that could be perceived as a potential conflict of interest.

Data availability

Data will be made available on request.

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References

Bally, I., Rossi, V., Lunardi, T., Thielens, N.M., Gaboriaud, C., Arlaud, G.J., 2009. Identification of the C1q-binding sites of human C1r and C1s: a refined three-dimensional model of the C1 complex of complement. J. Biol. Chem. 284 (29), 19340–19348. https://doi.org/10.1074/jbc.M109.004473.

Bally, I., Inforzato, A., Dalonneau, F., Stravalaci, M., Bottazzi, B., Gaboriaud, C., Thielens, N.M., 2019. Interaction of C1q with pentraxin 3 and IgM revisited: mutational studies with recombinant C1q variants. Front. Immunol. 10 https://doi.org/10.3389/fimmu.2019.00461.

van den Berg, R.H., Faber-Krol, M.C., Sim, R.B., Daha, M.R., 1998. The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells[1]. J. Immunol. 161 (12), 6924–6930. https://doi.org/10.4049/jimmunol.161.12.6924.

Carland, T.M., Gerwick, L., 2010. The C1q domain containing proteins: where do they come from and what do they do? Dev. Comp. Immunol. 34 (8), 785–790. https://doi.org/10.1016/j.dci.2010.02.014.

Carland, T.M., Locke, J.B., Nizet, V., Gerwick, L., 2012. Differential expression and intrachromosomal evolution of the sghC1q genes in zebrafish (*Danio rerio*). Dev. Comp. Immunol. 36 (1), 31–38. https://doi.org/10.1016/j.dci.2011.05.013.

Chen, M., Ding, M., Li, Y., Zhong, X., Liu, S., Guo, Z., Yin, X., Fu, S., Ye, J., 2018. The complement component 1 q (C1q) in Nile tilapia (*Oreochromis niloticus*): Functional characterization in host defense against bacterial infection and effect on cytokine response in macrophages. Dev. Comp. Immunol. 87, 98–108. https://doi.org/10.1016/j.dci.2018.05.023.

Chen, Y., Sha, Z., Wu, S., Chen, S., Zeng, Y., 2017. Does the different domain in sghC1q protein from *Cynoglossus semilaevis* perform functions independent? Gene Rep. 7, 59–63. https://doi.org/10.1016/j.genrep.2017.02.003.

Cui, Y., Okyere, S.K., Gao, P., Wen, J., Cao, S., Wang, Y., Deng, J., Hu, Y., 2021. *Ageratina adenophora* Disrupts the Intestinal Structure and Immune Barrier Integrity in Rats. Toxins 13 (9), 651. https://doi.org/10.3390/toxins13090651.

Daha, M.R., 2010. Role of complement in innate immunity and infections. Crit. Reviews™ Immunol. 30 (1), 47–52. https://doi.org/10.1615/CritRevImmunol.v30.i1.30.

Dawood, M.A.O., 2021. Nutritional immunity of fish intestines: important insights for sustainable aquaculture. Rev. Aquac. 13 (1), 642–663. https://doi.org/10.1111/raq.12492.

Del Valle, D.M., Kim Schulze, S., Huang, H.H., Beckmann, N.D., Nirenberg, S., Wang, B., Lavin, Y., Swartz, T.H., Madduri, D., Stock, A., Marron, T.U., Xie, H., Patel, M., Tuballes, K., Van Oekelen, O., Rahman, A., Kovatch, P., Aberg, J.A., Schadt, E., Jagannath, S., Mazumdar, M., Charney, A.W., Firpo-Betancourt, A., Mendu, D.R., Jhang, J., Reich, D., Sigel, K., Cordon Cardo, C., Feldmann, M., Parekh, S., Merad, M., Gnjatic, S., 2020. An inflammatory cytokine signature predicts COVID-19 severity and survival. Nat. Med. 26 (10), 1636–1643. https://doi.org/10.1038/s41591-020-1051-9.

Garcia, B.L., Zwarthoff, S.A., Rooijakkers, S.H.M., Geisbrecht, B.V., 2016. Novel evasion mechanisms of the classical complement pathway. J. Immunol. 197 (6), 2051–2060. https://doi.org/10.4049/jimmunol.1600863.

Ge, H.-L., Tan, K., Shi, L.-L., Sun, R., Wang, W.-M., Li, Y.-H., 2020. Comparison of effects of dsRNA and siRNA RNA interference on insulin-like androgenic gland gene (IAG) in red swamp crayfish *Procambarus clarkii*. Gene 752, 144783.

Ghai, R., Waters, P., Roumenina, L.T., Gadjeva, M., Kojouharova, M.S., Reid, K.B.M., Sim, R.B., Kishore, U., 2007. C1q and its growing family. Immunobiology 212 (4), 253–266. https://doi.org/10.1016/j.imbio.2006.11.001.

Huang, Y., Wang, W., Ren, Q., 2016. Identification and function of a novel C1q domain-containing (C1qDC) protein in triangle-shell pearl mussel (*Hyriopsis cumingii*). Fish. Shellfish Immunol. 58, 612–621. https://doi.org/10.1016/j.fsi.2016.10.010.

Huang, Y., Wu, L., Jin, M., Hui, K., Ren, Q., 2017. A C1qDC protein (HcC1qDC6) with three tandem C1q domains is involved in immune response of triangle-shell pearl mussel (*Hyriopsis cumingii*). Front. Physiol. 8, 521. https://doi.org/10.3389/fphys.2017.00521.

Jiang, S., Li, H., Zhang, D., Zhang, H., Wang, L., Sun, J., Song, L., 2015. A C1q domain containing protein from *Crassostrea gigas* serves as pattern recognition receptor and opsonin with high binding affinity to LPS. Fish. Shellfish Immunol. 45 (2), 583–591. https://doi.org/10.1016/j.fsi.2015.05.021.

Kishore, U., Ghebrehiwet, B., 2020. Editorial: C1q: a molecular bridge to innate and adaptive immunity. Front. Immunol. 11 https://doi.org/10.3389/fimmu.2020.00417.

Lee, S.Y., Söderhäll, K., 2001. Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*[1]. J. Immunol. 166 (12), 7319–7326. https://doi.org/10.4049/jimmunol.166.12.7319.

Li, J., Li, Q., Wu, Q., Gao, N., Wang, Z., Yang, Y., Shan, A., 2023. Exopolysaccharides of *Lactobacillus rhamnosus* GG ameliorate *Salmonella typhimurium*-induced intestinal inflammation via the TLR4/NF-κB/MAPK pathway. J. Anim. Sci. Biotechnol. 14 (1), 23. https://doi.org/10.1186/s40104-023-00830-7.

Li, M.-f, Zhang, H.-q, Sun, J.-s, 2023. A novel C1qDC (PoC1qDC) with a collagen domain in *Paralichthys olivaceus* mediates complement activation and against bacterial infection. Fish. Shellfish Immunol. 132, 108472 https://doi.org/10.1016/j.fsi.2022.108472.

Li, X.-q, Sun, H.-g, Wang, X.-h, Zhang, H.-j, Zhang, X.-s, Yu, Y., Liu, J., Guo, Q.-q, Yang, Z.-l, 2022. Activation of C3 and C5 may be involved in the inflammatory progression of PCM and GM. Inflammation 45 (2), 739–752. https://doi.org/10.1007/s10753-021-01580-2.

Li, Y., Niu, D., Bai, Y., Lan, T., Peng, M., Dong, Z., Li, J., 2019. Identification of a novel C1q complement component in razor clam *Sinonovacula constricta* and its role in antibacterial activity. Fish. Shellfish Immunol. 87, 193–201. https://doi.org/10.1016/j.fsi.2019.01.014.

Liang, X., Xiong, X., Cao, Y., Li, Z., Chen, J., Jiao, Y., Deng, Y., Du, X., 2022. Globular C1q domain-containing protein from *Pinctada fucata martensii* participates in the immune defense process. Fish. Shellfish Immunol. 123, 521–527. https://doi.org/10.1016/j.fsi.2022.02.048.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods (San. Diego, Calif.) 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Lukácsi, S., Mácsik-Valent, B., Nagy-Baló, Z., Kovács, K.G., Kliment, K., Bajtay, Z., Erdei, A., 2020. Utilization of complement receptors in immune cell–microbe interaction. FEBS Lett. 594 (16), 2695–2713. https://doi.org/10.1002/1873-3468.13743.

Lv, Z., Qiu, L., Wang, M., Jia, Z., Wang, W., Xin, L., Liu, Z., Wang, L., Song, L., 2018. Comparative study of three C1q domain containing proteins from pacific oyster *Crassostrea gigas*. Dev. Comp. Immunol. 78, 42–51. https://doi.org/10.1016/j.dci.2017.09.014.

Lv, Z., Wang, L., Jia, Z., Sun, J., Wang, W., Liu, Z., Qiu, L., Wang, M., Song, L., 2019. Hemolymph C1qDC promotes the phagocytosis of oyster *Crassostrea gigas* hemocytes by interacting with the membrane receptor β-integrin. Dev. Comp. Immunol. 98, 42–53. https://doi.org/10.1016/j.dci.2019.04.004.

Mei, J., Gui, J., 2008. Bioinformatic identification of genes encoding C1q-domain-containing proteins in zebrafish. J. Genet. Genom. 35 (1), 17–24. https://doi.org/10.1016/S1673-8527(08)60003-X.

Mei, J., Zhang, Q.-Y., Li, Z., Lin, S., Gui, J.-F., 2008. C1q-like inhibits p53-mediated apoptosis and controls normal hematopoiesis during zebrafish embryogenesis. Dev. Biol. 319 (2), 273–284. https://doi.org/10.1016/j.ydbio.2008.04.022.

Merle, N.S., Noe, R., Halbwachs-Mecarelli, L., Fremeaux-Bacchi, V., Roumenina, L.T., 2015. Complement System Part II: Role in Immunity. Front. Immunol. 6 https://doi.org/10.3389/fimmu.2015.00257.

Moreillon, P., Majcherczyk, P.A., 2003. Proinflammatory Activity of Cell-wall Constituents from Gram-positive Bacteria. Scand. J. Infect. Dis. 35 (9), 632–641. https://doi.org/10.1080/00365540310016259.

Nesargikar, P., Spiller, B., Chavez, R., 2012. The complement system: History, pathways, cascade and inhibitors. Eur. J. Microbiol. Immunol. 2 (2), 103–111. https://doi.org/10.1556/eujmi.2.2012.2.2.

Oosterhoff, J.J., Larsen, M.D., van der Schoot, C.E., Vidarsson, G., 2022. Afucosylated IgG responses in humans–structural clues to the regulation of humoral immunity. Trends Immunol. 43 (10), 800–814. https://doi.org/10.1016/j.it.2022.08.001.

Palomino Ramos, A.R., Campelo, D.A.V., Carneiro, C.Ld.S., Zuanon, J.A.S., da Matta, S.L. P., Furuya, W.M., Salaro, A.L., 2022. Optimal dietary L-glutamine level improves growth performance and intestinal histomorphometry of juvenile giant trahira (*Hoplias lacerdae*), a Neotropical carnivorous fish species. Aquaculture 547, 737469. https://doi.org/10.1016/j.aquaculture.2021.737469.

Pei, G., Liu, G., Pan, X., Pang, Y., Li, Q., 2016. L-C1qDC-1, a novel C1q domain-containing protein from *Lethenteron camtschaticum* that is involved in the immune response. Dev. Comp. Immunol. 54 (1), 66–74. https://doi.org/10.1016/j.dci.2015.08.011.

Peronato, A., Minervini, G., Tabarelli, M., Ballarin, L., Franchi, N., 2021. Characterisation and functional role of a novel C1qDC protein from a colonial ascidian. Dev. Comp. Immunol. 122, 104077 https://doi.org/10.1016/j.dci.2021.104077.

Qiang, J., Tao, Y.-F., He, J., Li, H.-X., Xu, P., Bao, J.-W., Sun, Y.-L., 2017. Inhibition of miR-92d-3p enhances inflammation responses in genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) with Streptococcus iniae infection by modulating complement C3. Fish. Shellfish Immunol. 63, 367–375. https://doi.org/10.1016/j.fsi.2017.02.036.

Song, X., Zhao, J., Bo, Y., Liu, Z., Wu, K., Gong, C., 2014. Aeromonas hydrophila induces intestinal inflammation in grass carp (*Ctenopharyngodon idella*): an experimental model. Aquaculture 434, 171–178. https://doi.org/10.1016/j.aquaculture.2014.08.015.

Tang, X., Arora, G., Matias, J., Hart, T., Cui, Y., Fikrig, E., 2022. A tick C1q protein alters infectivity of the Lyme disease agent by modulating interferon γ. Cell Rep. 41 (8), 111673 https://doi.org/10.1016/j.celrep.2022.111673.

Taylor, S.R., Ramsamooj, S., Liang, R.J., Katti, A., Pozovskiy, R., Vasan, N., Hwang, S.-K., Nahiyaan, N., Francoeur, N.J., Schatoff, E.M., Johnson, J.L., Shah, M.A., Dannenberg, A.J., Sebra, R.P., Dow, L.E., Cantley, L.C., Rhee, K.Y., Goncalves, M.D., 2021. Dietary fructose improves intestinal cell survival and nutrient absorption. Nature 597 (7875), 263–267. https://doi.org/10.1038/s41586-021-03827-2.

Vadászi, H., Kiss, B., Micsonai, A., Schlosser, G., Szaniszló, T., Kovács, R.Á., Györffy, B. A., Kékesi, K.A., Goto, Y., Uzonyi, B., Liliom, K., Kardos, J., 2022. Competitive inhibition of the classical complement pathway using exogenous single-chain C1q recognition proteins. J. Biol. Chem. 298 (7), 102113 https://doi.org/10.1016/j.jbc.2022.102113.

Vargas-Albores, F., Martínez-Córdova, L.R., Hernández-Mendoza, A., Cicala, F., Lago-Lestón, A., Martínez-Porchas, M., 2021. Therapeutic modulation of fish gut microbiota, a feasible strategy for aquaculture? Aquaculture 544, 737050. https://doi.org/10.1016/j.aquaculture.2021.737050.

Vecchiarelli, A., Retini, C., Casadevall, A., Monari, C., Pietrella, D., Kozel, T.R., 1998. Involvement of C3a and C5a in interleukin-8 secretion by human polymorphonuclear cells in response to capsular material of *Cryptococcus neoformans*. Infect. Immun. 66 (9), 4324–4330. https://doi.org/10.1128/iai.66.9.4324-4330.1998.

Vowels, B.R., Yang, S., Leyden, J.J., 1995. Induction of proinflammatory cytokines by a soluble factor of Propionibacterium acnes: implications for chronic inflammatory acne. Infect. Immun. 63 (8), 3158–3165. https://doi.org/10.1128/iai.63.8.3158-3165.1995.

Wang, J., Thaimuangphol, W., Chen, Z., Li, G., Gong, X., Zhao, M., Chen, Z., Wang, B., Wang, Z., 2022. A C1q domain-containing protein in *Pinctada fucata* contributes to the innate immune response and elimination of the pathogen. Fish. Shellfish Immunol. 131, 582–589. https://doi.org/10.1016/j.fsi.2022.10.031.

Wang, L., Wang, L., Kong, P., Yang, J., Zhang, H., Wang, M., Zhou, Z., Qiu, L., Song, L., 2012. A novel C1qDC protein acting as pattern recognition receptor in scallop *Argopecten irradians*. Fish. Shellfish Immunol. 33 (2), 427–435. https://doi.org/10.1016/j.fsi.2012.05.032.

Wang, L., Wang, L., Zhang, D., Jiang, Q., Sun, R., Wang, H., Zhang, H., Song, L., 2015. A novel multi-domain C1qDC protein from zhikong scallop *Chlamys farreri* provides new insights into the function of invertebrate C1qDC proteins. Dev. Comp. Immunol. 52 (2), 202–214. https://doi.org/10.1016/j.dci.2015.05.009.

Wu, M., Jia, B.B., Li, M.F., 2022. Complement C3 and activated fragment C3a are involved in complement activation and anti-bacterial immunity. Front. Immunol. 13, 813173 https://doi.org/10.3389/fimmu.2022.813173.

Xiong, X., Li, C., Zheng, Z., Du, X., 2021. Novel globular C1q domain-containing protein (PmC1qDC-1) participates in shell formation and responses to pathogen-associated molecular patterns stimulation in *Pinctada fucata martensii*. Sci. Rep. 11 (1), 1105. https://doi.org/10.1038/s41598-020-80295-0.

Yang, L., Liu, X., Liu, W., Li, X., Qiu, L., Huang, J., Jiang, S., 2013. Characterization of complement 1q binding protein of tiger shrimp, *Penaeus monodon*, and its C1q binding activity. Fish. Shellfish Immunol. 34 (1), 82–90. https://doi.org/10.1016/j.fsi.2012.10.002.

Zeng, Y., Xiang, J., Lu, Y., Chen, Y., Wang, T., Gong, G., Wang, L., Li, X., Chen, S., Sha, Z., 2015. sghC1q, a novel C1q family member from half-smooth tongue sole (*Cynoglossus semilaevis*): identification, expression and analysis of antibacterial and antiviral activities. Dev. Comp. Immunol. 48 (1), 151–163. https://doi.org/10.1016/j.dci.2014.09.010.

Zhang, H., Song, L., Li, C., Zhao, J., Wang, H., Qiu, L., Ni, D., Zhang, Y., 2008. A novel C1q-domain-containing protein from Zhikong scallop *Chlamys farreri* with lipopolysaccharide binding activity. Fish. Shellfish Immunol. 25 (3), 281–289. https://doi.org/10.1016/j.fsi.2008.06.003.

Zhang, Y., Li, C.-N., Jiang, W.-D., Wu, P., Liu, Y., Kuang, S.-Y., Tang, L., Li, S.-W., Jin, X.-W., Ren, H.-M., 2022. An emerging role of vitamin D3 in amino acid absorption in different intestinal segments of on-growing grass carp (*Ctenopharyngodon idella*). Anim. Nutr. 10, 305–318. https://doi.org/10.1016/j.aninu.2022.05.004.

Zhou, L., Wei, J.-f, Lin, K.-t, Gan, L., Wang, J.-j, Sun, J.-j, Xu, X.-p, Liu, L., Huang, X.-d, 2020. Intestinal microbial profiling of grass carp (*Ctenopharyngodon idellus*) challenged with *Aeromonas hydrophila*. Aquaculture 524, 735292. https://doi.org/10.1016/j.aquaculture.2020.735292.

Zhou, Y., Guo, M., Li, Y., Zhang, Y., Xu, W., Zhao, D., Chen, Y., Zuo, A., Qu, F., Tang, J., Cao, S., Liu, Z., 2022. Effects of Ghrelin on intestinal cell proliferation, the expression of protein absorption and metabolism factors in juvenile grass carp (*Ctenopharyngodon idella*). Aquac. Rep. 22, 100928 https://doi.org/10.1016/j.aqrep.2021.100928.

Zhu, L., Gao, Y., Yuan, G., Jiang, X., Zhang, J., Zhao, X., Pei, C., Li, L., Kong, X., 2021. Identification and immune functional analysis of a novel C1q domain-containing protein in Yellow River Carp (*Cyprinus carpio L.*). Aquaculture 534, 736313. https://doi.org/10.1016/j.aquaculture.2020.736313.

Zong, Y., Liu, Z., Wu, Z., Han, Z., Wang, L., Song, L., 2019. A novel globular C1q domain containing protein (C1qDC-7) from *Crassostrea gigas* acts as pattern recognition receptor with broad recognition spectrum. Fish. Shellfish Immunol. 84, 920–926. https://doi.org/10.1016/j.fsi.2018.10.079.