Fish and Shellfish Immunology 88 (2019) 189–197

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Complement component 3 (C3): An important role in grass carp (*Ctenopharyngodon idella*) experimentally exposed to *Aeromonas hydrophila*

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ARTICLE INFO

Keywords:
Complement component 3
Aeromonas hydrophila
Histological alterations
Liver index
Grass carp

ABSTRACT

Complement is traditionally recognized as part of the innate immune system, defending the host against the invasion of foreign pathogens. In complement system, C3 (complement component 3) is a central component. Therefore, research into C3 can help us better understand the functions of fish complement system. In this study, we detected the grass carp C3 (*gcC3*) mRNA expression in all sample tissues from healthy grass carp, which was highest in the liver, followed by the heart and the spleen, and lowest in the muscle, head kidney, trunk kidney, blood and intestine. After infection with *Aeromonas hydrophila*, *gcC3* mRNA expression levels were significantly upregulated in the gill, liver, spleen, intestine, trunk kidney and head kidney. Interestingly, C3 protein levels were downregulated and subsequently upregulated in the liver and serum. Histologically, C3 protein at 24 h pi was over expressed in necrotic liver sites, and the liver index (LI) at this point was significantly higher than that of the control. These findings are indicated that C3 plays an important role in the immune response of grass carp after *A. hydrophila* infection, and C3 protein may play an assistant role in repairing liver tissues from *A. hydrophila* injury.

1. Introduction

The grass carp, *Ctenopharyngodon idella*, is a native Chinese freshwater fish with broad distribution from the catchment area of the Pearl River in southern China to that of the Heilongjiang River in northern China [1]. Due to its good-quality meat, excellent growth performance, and wide adaptability, the grass carp has been transplanted to the rest of the world, especially Asia [2]. In 2014, the global grass carp production reached about 5.5 million tons [3], and it became a significant economic freshwater species [4]. However, behind the rapid development of grass carp farming, high disease incidence and mortality due to bacterial septicemia has become a serious concern, and is mainly caused by *Aeromonas hydrophila* [5].

Complement, a highly sophisticated defense system, playing a pivotal role in defense against pathogen infection [6]. Complement is activated by three major pathways: the classical, the alternative, and the lectin pathways, all of which converge on complement component 3 (C3), which ultimately drives complement effector functions to eliminate the invading pathogens, regulate the adaptive immune [7,8], and modify the self cells, such as apoptotic cells and cellular debris, to protect against autoimmunity [9–12]. Moreover, C3, this versatile and flexible molecule, also interacts with various molecules to perform other functions, such as cell survival, growth, and differentiation in various tissues [13,14]. Our laboratory has focused effort on the role of C3 in injured liver tissues of grass carp.

Hepatocytes represent the major source of most plasma complement proteins, especially C3 [15], but modern molecular biological methods have confirmed that C3 are synthesized at multiple sites [16]. In rainbow trout (*Oncorhynchus mykiss*), the ontogenic appearance and mapping of extrahepatic synthesis of complement components have revealed widespread production of several complement components [17]. And studies on Atlantic halibut (*Hippoglossus hippoglossus*) and

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https://doi.org/10.1016/j.fsi.2019.02.061
Received 30 December 2018; Received in revised form 22 February 2019; Accepted 25 February 2019
Available online 01 March 2019
1050-4648/

Atlantic cod (*Gadus morhua*) have revealed the presence of C3 mRNA and proteins in several tissues post-hatch [18–20]. Moreover, some studies have researched the relationship between serum C3 levels and human disease at different periods, and have shown that, as an indicator of humoral immunity levels, serum C3 levels are of great significance for clinical diagnosis and treatment [21,22]. Recently, Markiewski et al. have indicated that the third component of complement (C3) is critical for normal liver recovery after toxic injury [23]. All in all, C3 as an important molecule has attracted much attention. In some teleost fish, the analysis of characterization and expression of C3 have been reported [24–26], including the full-length cDNA and simple expression of *gcC3*. However, studies on the functional aspects of C3, especially those outside of immune function, have not been in-depth enough.

In our study, we detected *gcC3* mRNA expression in various tissues, C3 protein expression in liver tissue site, and intrahepatic or serous C3 levels of grass carp; and evaluated the histological change of the liver in grass carp infected with *A. hydrophila*. Moreover, we further analyzed the possible relationship between the liver indexes and the expression levels of C3 mRNA or C3 protein, and investigated the changes of C3 expression sites in injured liver tissues. Our results not only aid understanding of the role of C3 in grass carp against bacterial infection, but also provide some help in disease diagnosis of fish. All of them help us understand the functions of C3 in fish.

2. Materials and methods

2.1. Sequence analysis of gcC3

The *gcC3* complementary DNA (cDNA) sequence was obtained from NCBI GenBank (https://www.ncbi.nlm.nih.gov/nuccore/; accession number: AY374472.1). Primer Premier 5.0 software (https://www.premierbiosoft.com/) was used to design primers. ExPASy (http://web.expasy.org/protparam/) was used to analyze the amino acid sequence. The open reading frame (ORF) was searched using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). The homology of *gcC3* cDNA and the theoretical protein sequences was analyzed using the National Center for Biotechnology Information BLASTp and BLASTn (http://www.ncbi.nlm.nih.gov/).

2.2. Experimental animals

Grass carp individuals (n = 150, 1-year-old) were obtained from the Center of Grass Carp Breeding, Jiangsu, China. The fish were transferred from the farm to a laboratory sterilized circulating water system containing aerated water and were acclimated for two weeks. The water temperature, pH, dissolved oxygen and total ammonia were kept at 28 ± 0.5 °C, 7.4 ± 0.4, 7.8 ± 0.8 mg/L, and < 0.01 mg/L, respectively. All fish were fed twice daily (morning and late afternoon) with 5% of their total biomass. The weight of the fish at the start of the experiment was 39.8 ± 5.4 g. The fish were handled according to the guidelines on the care and use of animals for scientific purposes established by the Institutional Animal Care and Use Committee (IACUS) of Shanghai Ocean University, Shanghai, China.

2.3. Acute infection of A. hydrophila

Total of 150 fish were randomly divided into six groups (25 individuals per group, specific density < 1 g fish/L), with three groups as experimental groups and other three as control groups. For the experimental groups, the *A. hydrophila* (AH10; Aquatic Pathogen Collection Center of Ministry of Agriculture, Shanghai, China) concentration was adjusted to 2.4×10^7 colony-forming units (cfu)/ml (median lethal concentration), and the experimental groups were injected intraperitoneally with *A. hydrophila* at a dose of 2.4×10^6 cells suspended in 100 μL phosphate-buffered saline (PBS) per fish. The control groups were injected with the same volume of PBS per fish. Careful operation throughout the experiment to prevent grass carp from stress response.

2.4. Sample preparation

Sampling was performed at 0, 4, 8, 12, 24, 48, and 72 h post-injection (pi), respectively, and 2 fish from every group was taken each time. The fish were firstly anaesthetized with tricaine methanesulfonate (MS-222; Sigma, USA) and then their blood was obtained immediately taken from the fish caudal vein for serum preparation. After that, the fish were dissected, and tissues were rapidly isolated. Twelve tissues (gill, liver, spleen, intestine, trunk kidney, head kidney, heart, skin, muscle, brain, blood, fin) from healthy fish were sampled for *gcC3* distribution profile analysis. For the time-dependent expression profile analysis of *gcC3*, six immune-related tissues (gill, liver, spleen, intestine, trunk kidney, head kidney) were collected at six time points post-injection (pi). Above samples were immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction. In addition, small sections of liver tissue were fixed in 4% paraformaldehyde solution for the preparation of paraffin section or submerged in cold PBS (0.01 M, pH 7.4) for the production of tissue homogenate.

Whole-blood samples were allowed to clot at 4 °C overnight, followed by centrifuged (1000 × *g*, 4 °C) for 20 min. Finally, the supernatant (serum) samples of each group were mixed with equal volume, and then stored at −80 °C.

The liver was removed from cold PBS, dried by absorbent paper, weighted, minced into small pieces, and homogenized to a 1/10 (w/v) ratio in cold PBS containing trypsin inhibitor (Solarbio, China) with a glass homogenizer on ice. Then each homogenate was centrifuged at 5000 × *g* for 5 min at 4 °C. The supernatants of each group were mixed at the same volume, and then stored at −80 °C.

All paraformaldehyde-fixed liver tissues were dehydrated, cleared, embedded in paraffin, cut into sections 4-5 μm thick and stained with immunohistochemical studies.

2.5. Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA purity and integrity were determined by measuring the OD value (used a NanoDrop 2000C spectrophotometer, USA) and agarose gel electrophoresis, respectively. After RNA quality detection, the total RNA samples of each group were mixed at the same concentration.

Total RNA of each specimen (1 μg) was reverse-transcribed using RNase-free gDNA (genomic DNA) Eraser (TaKaRa, Japan); a five-fold dilution series of the cDNAs was used to construct standard curves using the CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). NovoStart SYBR qPCR SuperMix (Novoprotein, China) was used to perform the qRT-PCR. The β-actin gene (GenBank accession number: DQ211096.1) was used as the internal reference gene [27]. The qRT-PCR cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. The dissociation curve for each gene was created for proving a single PCR product. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) value [28]. Differences in the data from the control and experimental groups were assessed using one-way analysis of variance (ANOVA) with SPSS 22.0; $P < 0.05$ was considered statistically significant. Table 1 lists the primers used in this study.

2.6. Assays for complement C3

Following method of Li et al. [29], the contents of complement C3 in fish serum and liver were determined by using the kits from the Hengyuan Ltd., Shanghai, China, according to the manufacturer's

Table 1
Primer sequences used in the study.

Primer	Sequence (5′–3′)	Application
gcβ-actin-F	TCCTTCTTGGGTATGGAGTCTTG	qRT-PCR
gcβ-actin-R	CAGAGTATTTACGCTCAGGTGGG	qRT-PCR
gcC3-F	CCGTCCTTCCGCTTCGTG	qRT-PCR
gcC3-R	CCTCCTGCGGTGTGCGAC	qRT-PCR

instructions.

2.7. Liver C3 expression study by immunohistochemical (IHC) methods

The liver slides were deparaffinized and rehydrated, then incubated in 3% hydrogen peroxide (Weiao Ltd., Shanghai, China) for 10 min, washed in PBS, and incubated for 10 min in antigen retrieval buffers (Weiao) at 95 °C. Slides were cooled to room temperature, then blocked by incubation in 5% BSA at room temperature for 35 min. After that, Polyclonal anti-grass carp C3 (GLS Ltd., Shanghai, China – diluted 1:100 in 0.01 M PBS) was used as primary antibodies. The slides were incubated for 18–22 h at 4 °C, washed in PBS, incubated with biotinylated goat anti-mouse and anti-rabbit (Link-DAKO, California, USA) for 35 min at 37 °C, washed once more with PBS and incubated with streptavidin biotin-peroxidase complex (Weiao) for 20 min at room temperature. The reaction was terminated with PBS, and stained with DAB Horseradish Peroxidase Color Development Kit (Weiao). The slides were counterstained with Harris's hematoxylin, dehydrated, cleared and mounted with coverslips.

2.8. Histological alterations

The liver slides were prepared for histological analysis using standard methods [30] and stained with hematoxylin and eosin (H&E) using a rapid H&E staining protocol [31].

The histological alterations were quantified according to the semi-quantitative system proposed by Bernet et al. [32]. The system classifies histological changes into five reaction patterns: 1) circulatory disturbance (CD); 2) regressive alterations (RC); 3) progressive alterations (PC); 4) inflammation (I); and 5) tumors (T). For each reaction pattern, the system considers several alterations to assess the histological alterations and uses a score ranging from 0 (unchanged) to 6 (severe/diffuse occurrence) to quantify each change. For each change, an importance factor ranging from 1 (minimal pathological importance) to 3 (marked pathological importance) is attributed. Next, the score is multiplied by the importance factor to obtain the final value for each change. The sum of these final values for one reaction pattern is used to estimate the CD index (ICD), RC index (IRC), PC index (IPC), I index (II), T index (IT), and liver index (LI). The LI values were used to classify the severity of the histological alterations using the classification system by Zimmerli et al. [33]: Class I ($LI < 10$), normal tissue structure with minor histological alterations; Class II ($10 \leq LI < 20$), normal tissue structure with moderate histological alterations; Class III ($20 \leq LI < 30$), moderate modifications of normal tissue; Class IV ($30 \leq LI < 40$), pronounced histological changes in the liver; and Class V ($LI \geq 40$), severe histological alterations of the liver. Table 2 lists the assessment tools used for the liver histological alterations as per Bernet et al. [32].

2.9. Statistical analysis

IBM SPSS 22.0 Software (IBM, USA) was used for data analysis, and statistical significance was defined as $P < 0.05$. All C3 level experiments were performed in triplicate; significant differences among samples were determined by one-way ANOVA. Significant differences between the histological index for each time point were compared using a nonparametric Kruskal–Wallis test, followed by multiple comparisons.

3. Results

3.1. Tissue distribution of gcC3 in healthy individuals

gcC3 mRNA was expressed in twelve tissues from healthy grass carp. The expression of *gcC3* was highest in the liver, which was more 70,000-fold higher than in the intestine ($P < 0.01$; Fig. 1). Transcripts were also abundant in heart and spleen, but weakly expressed in the gill, muscle, head kidney, trunk kidney, blood, and intestine ($P > 0.05$; Fig. 1).

3.2. gcC3 expression upon bacterial challenge in immune tissues

For time-dependent expression profiling of *gcC3* mRNA in the major immune organs, we sampled the gill, liver, spleen, intestine, trunk kidney, and head kidney tissues at six time points following injection with *A. hydrophila* (Fig. 2). In the liver, *gcC3* mRNA expression increased significantly between 4 h and 12 h pi ($P < 0.01$) but decreased significantly between 24 h and 72 h pi compared to the control ($P < 0.05$; Fig. 2A). In the spleen, *gcC3* mRNA expression was significantly upregulated between 4 h and 8 h pi ($P < 0.01$), and then was downregulated between 12 h and 72 h pi to the normal levels measured in the control ($P < 0.01$; Fig. 2B). In the gill, significant changes in expression occurred between 4 h and 12 h pi after *A. hydrophila* challenge, and the highest value was measured at 8 h pi ($P < 0.01$; Fig. 2C). In the head kidney, *gcC3* mRNA expression levels changed dramatically between 4 h and 12 h pi, the highest value was measured at 4 h pi ($P < 0.01$; Fig. 2D). In the trunk kidney, *A. hydrophila* challenge resulted in significantly increased *gcC3* mRNA expression, which peaked at 12 h pi ($P < 0.01$; Fig. 2E), followed by returned to normal expression levels. In the intestine, there was no significant change at 4 h and 8 h pi ($P > 0.05$), but expression increased significantly between 8 h and 12 h pi ($P < 0.01$; Fig. 2F).

3.3. Intrahepatic or serous C3 protein levels

Liver *C3* protein level continued to be downregulated after 4 h pi and reached a nadir at 8 h pi ($P < 0.05$; Fig. 3A), and was then upregulated at 12 h pi ($P < 0.05$). However, the levels were maintained at normal levels at 24–48 h pi compared to the control ($P > 0.05$). Finally, *C3* level increased significantly to peak at 72 h pi ($P < 0.01$).

Serum *C3* protein level was significantly lower in the experimental group than in the control at 12 h pi ($P < 0.05$; Fig. 3B). However, the result was diametrically opposed at 48 h and 72 h pi ($P < 0.01$). No statistically significant difference in *C3* levels were observed at the other time points ($P > 0.05$).

3.4. Immunohistochemical (IHC) methods for analyzing C3 expression in liver tissue

By immunohistochemical staining, we detected a lot of positive cells in the liver during infection (Fig. 4). In control groups, C3 protein was observed around the nuclei of hepatocytes (Fig. 4A). At 4–8 h pi, C3 protein was mainly found in cytoplasm of hepatic cells (Fig. 4B and C). At 12 h pi, there were a large number of positive cells in the liver, and C3 protein was mainly distributed in the cytoplasm of hepatocytes (Fig. 4D). At 24 h pi, we found that C3 protein was abundant in damaged intercellular spaces (Fig. 4E1) and some amount in blood vessels (Fig. 4E2). At 48 h pi, C3 protein was observed in cytoplasm and around the nuclei of hepatocytes (Fig. 4F). At 72 h pi, we found that a large amount of C3 protein in the cytoplasm and some amount around the blood cells (Fig. 4G).

Table 2
Histopathological assessment tools for grass carp liver.

Reaction pattern (rp)	Functional unit of tissue	Alteration (alt)	Importance factor (W)	Score value (a)	Index (I)
Circulatory disturbances		Hemorrhage/hyperemia/aneurysm	$W_{LC1} = 1$	aLC1	I_{LC}
		Intercellular edema	$W_{LC2} = 1$	aLC2	
Regressive changes	Liver tissue	Architectural and structural alterations	$W_{LR1} = 1$	aLR1	I_{LR}
		Plasma alterations	$W_{LR2} = 1$	aLR2	
		Deposits	$W_{LR3} = 1$	aLR3	
		Nuclear alterations	$W_{LR4} = 2$	aLR4	
		Atrophy	$W_{LR5} = 2$	aLR5	
		Necrosis	$W_{LR6} = 3$	aLR6	
		Vacuolar degeneration			
	Interstitial tissue	Architectural and structural alterations	$W_{LR7} = 1$	aLR7	
		Plasma alterations	$W_{LR8} = 1$	aLR8	
		Deposits	$W_{LR9} = 1$	aLR9	
		Nuclear alterations	$W_{LR10} = 2$	aLR10	
		Atrophy	$W_{LR11} = 2$	aLR11	
		Necrosis	$W_{LR12} = 3$	aLR12	
	Bile duct	Architectural and structural alterations	$W_{LR13} = 1$	aLR13	
		Plasma alterations	$W_{LR14} = 1$	aLR14	
		Deposits	$W_{LR15} = 1$	aLR15	
		Nuclear alterations	$W_{LR16} = 2$	aLR16	
		Atrophy	$W_{LR17} = 2$	aLR17	
		Necrosis	$W_{LR18} = 3$	aLR18	
Progressive changes	Liver tissue	Hypertrophy	$W_{LP1} = 1$	aLP1	I_{LP}
		Hyperplasia	$W_{LP2} = 2$	aLP2	
	Interstitial tissue	Hypertrophy	$W_{LP3} = 1$	aLP3	
		Hyperplasia	$W_{LP4} = 2$	aLP4	
	Bile duct	Hypertrophy	$W_{LP5} = 1$	aLP5	
		Hyperplasia	$W_{LP6} = 2$	aLP6	
		Wall proliferation of bile ducts or ductules			
Inflammation		Exudate	$W_{LI1} = 1$	aLI1	I_{LI}
		Activation of RES	$W_{LI2} = 1$	aLI2	
		Infiltration	$W_{LI3} = 2$	aLI3	
Tumor		Benign tumor	$W_{LT1} = 2$	aLT1	I_{LT}
		Malignant tumor	$W_{LT2} = 3$	aLT2	

An importance factor ($W_{L\ rp\ alt}$) of 1–3 is assigned to each alteration, which is composed of the reaction pattern (rp) and the alteration (alt). The score must be rated for every alteration with a score of 0–6. The addition of supplementary alterations can be achieved according to the specific needs. However, these should not be considered for the index calculation.

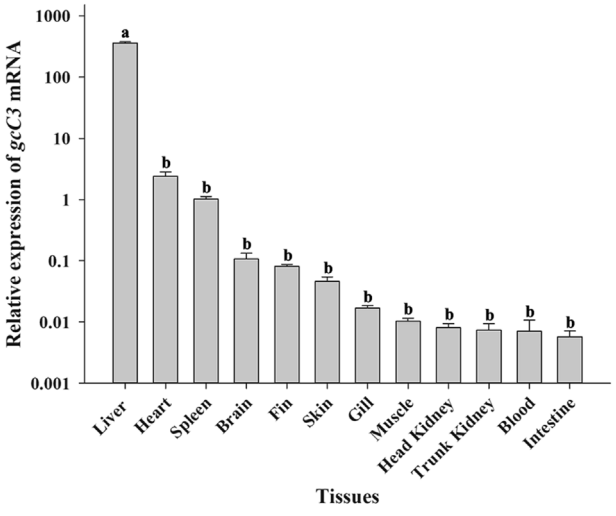

Fig. 1. Expression of *gcC3* mRNA in twelve healthy tissues of grass carp determined by RT-PCR. The mRNA expression levels of all tissues are expressed relative to which of the spleen, and the relative expression was calculated and normalized using the β-actin mRNA level. Data represent the mean ± SD of individual RNA samples (n = 3). Different letters indicate a statistically significant difference compared to controls: $P < 0.05$.

3.5. Histological alterations in the liver

Normal structure and systematic arrangement of hepatocytes were observed in the control. In addition, the nuclei were located in the center in most hepatocytes; however, mild hyperemia was observed in some samples (Fig. 5A). At 4 h pi, there was minor dilation of the hepatic cords and sinusoids, and slight hemorrhage and necrosis were observed in the liver tissue (Fig. 5B). At 8 h pi, some hyperemia, partial necrosis of hepatocytes, partial hepatic cord dissolution, and hepatic sinusoid dilatation were observed (Fig. 5C). At 12 h pi, the subsequent necrosis changed to bridge-like necrosis, and moderate hyperemia persisted. During this period, the partial hepatocytic wall disappeared and the hepatocytes became irregular in shape (Fig. 5D). At 24 h pi, hemorrhage, necrosis, and hyperemia became substantially more aggravated. The partial connections between the vessels and hepatocytes were completely fractured. In addition, inflammatory cell infiltration became more obvious compared to that observed earlier (Fig. 5E1, 5E2). At 48 h pi, hyperemia and necrosis were alleviated; moreover, the connections between the vessels and hepatocytes were whole (Fig. 5F). At 72 h pi, the livers of the surviving grass carp had near normal hepatocyte morphology and orderly cell arrangement. However, they still exhibited a minor amount of hemorrhage and hyperemia, accompanied by mild inflammatory cell infiltration (Fig. 5G).

4. Discussion

The complement system is a complex group of proteins and glycoproteins that serves as both an innate and acquired defense against bacterial infection [34]. So far, the immune function of many complement components has been studied in grass carp [35–37], but there are rare studies on functions other than immunity. Therefore, the present research takes C3, an important complement molecule, as the research object to explore its function in grass carp.

A previous report has indicated differential C3 expression across

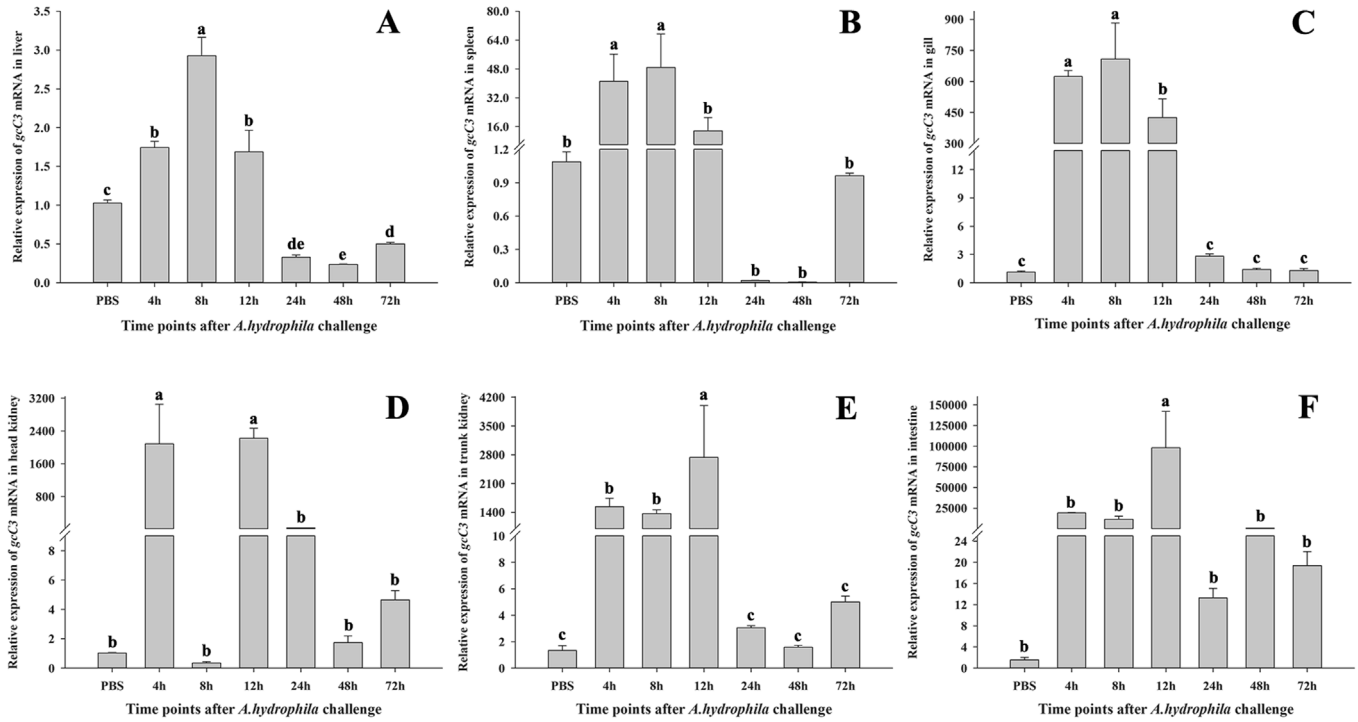

Fig. 2. Expression of *gcC3* mRNA in liver (A), spleen (B), gill (C), head kidney (D), trunk kidney (E), and intestine (F) tissues after injection with *A. hydrophila*. Injection with PBS was used as a control. Relative expression was calculated and normalized according to β-actin mRNA expression. Data represent the mean ± SD (n = 3). Different letters indicate a statistically significant difference compared to controls: $P < 0.05$.

tissues [24]. In the present study, *gcC3* transcript expression was highest in the liver, and expression differed in the other 11 tissues (Fig. 1). This is in agreement with previous reports on mammal and teleost C3. In mammals, C3 is primarily expressed in the liver, with secondary sites spanning a variety of tissues, including the central nervous system, and gastrointestinal, reproductive, and lymphoid organs [34,38,39]. Furthermore, the liver is generally considered the prime organ involved in C3 synthesis in many teleosts, such as large yellow croaker (*Larimichthys crocea*) [25], rohu (*Labeo rohita*) [24], and common carp (*Cyprinus carpio*) [40]. Extrahepatic expression of C3 has been reported in many species, including rainbow trout (*Oncorhynchus mykiss*) [41], orange-spotted grouper (*Epinephelus coioides*) [42], common carp (*C. carpio*) [43], European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) [44]. In grass carp, the *gcC3* tissue expression profiles suggest that both local and systemic signaling pathways are initiated in the immune and non-immune tissues of healthy fish.

In the present study, we investigated *gcC3* mRNA expression in the liver, spleen, gill, head kidney, trunk kidney, and intestine tissue of grass carp following inoculation with *A. hydrophila* (Fig. 2). As shown by Fig. 2, multiple tissues regulate *gcC3* mRNA expression levels, and the profiles also indicated that the *A. hydrophila* infection was initiated at 4 h pi and terminated between 12 h and 24 h pi. The *gcC3* mRNA expression in different tissues showed an overall trend for rapid upregulated early expression (Fig. 2). This phenomenon indicated that despite the liver is the main organ for synthesis of C3, other tissues can assist in the synthesis of C3 for combating bacterial infection. Previous studies have noted that C3 transcription was upregulated in the liver, spleen, and brain of *L. crocea* after infection with *Vibrio alginolyticus* [25], was drastically upregulated in the spleen of rainbow trout with *Ichthyophthirius multifiliis* infection [41], and was upregulated in the liver and kidney of *Labeo rohita* after *A. hydrophila* infection [24]. After 24 h pi, the expression of *gcC3* mRNA returned to normal levels except in the liver, in which the expression level was down to 0.5 times of the

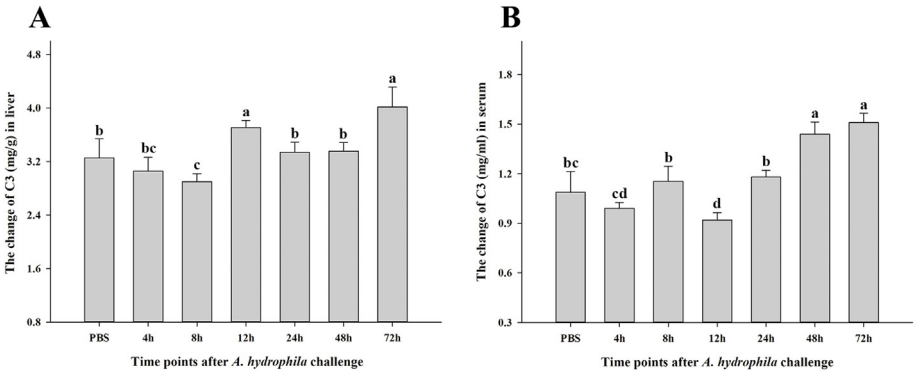

Fig. 3. Liver C3 (mg/g) levels (A) and serum C3 (mg/ml) levels (B) after injection with *A. hydrophila*. Injection with PBS was used as a control. The data represent the mean ± SD (n = 3). Different letters indicate a statistically significant difference between different sampled times: $P < 0.05$.

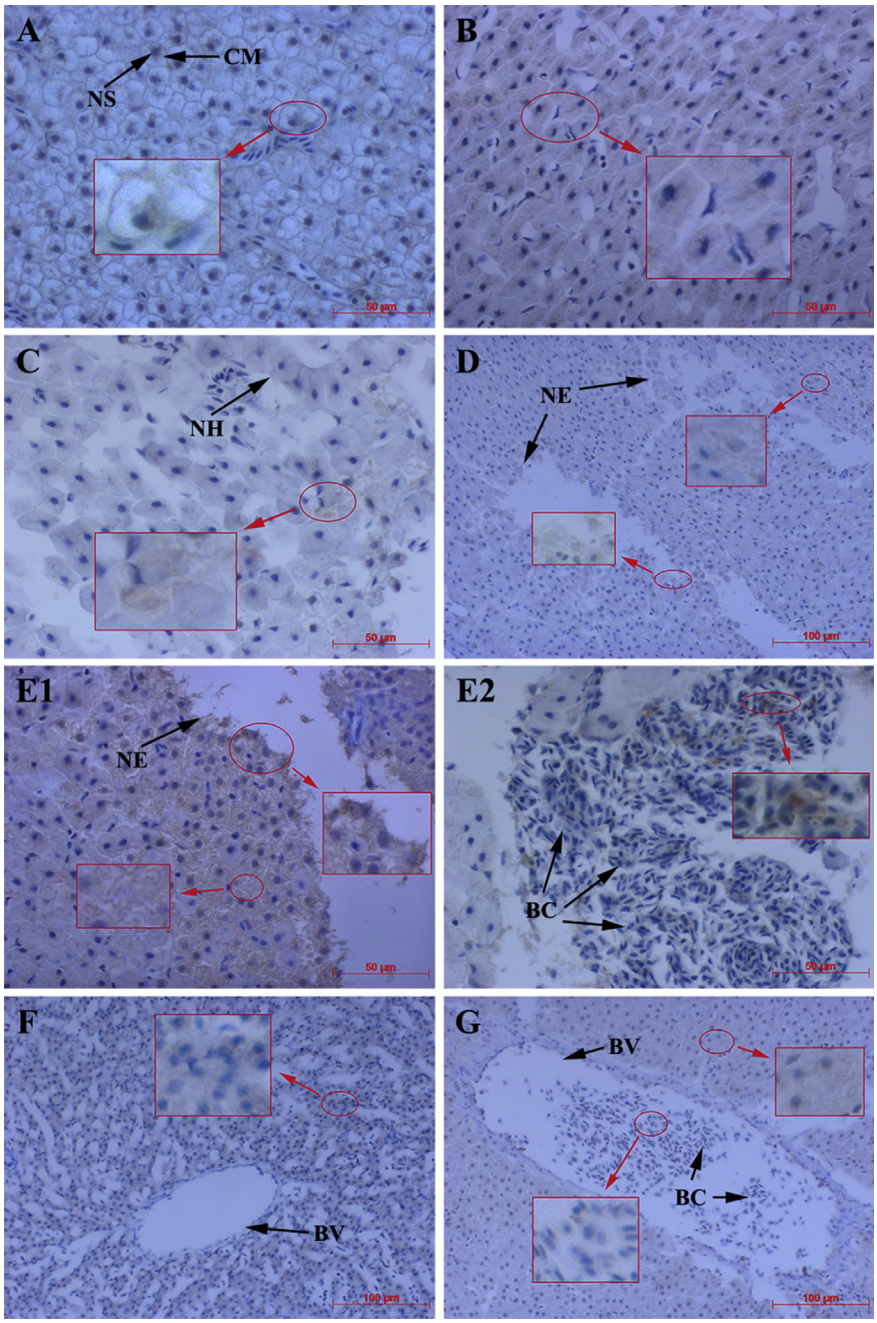

Fig. 4. Presence of complement C3 in liver tissues as revealed by immunohistochemical staining. The nucleus (NS) is stained blue with hematoxylin. The cytoplasm (CM) is colorless. Antibody staining is shown in brown from DAB plus substrate. (A) C3 protein expression in normal structure of hepatocytes (NH) (scale bar = 50 μm, × 40 magnification). (B) C3 protein expression in minor dilation of the hepatic cords at 4 h pi (scale bar = 50 μm, × 40 magnification). (C) C3 protein expression in partial necrosis (NE) of hepatocytes, partial hepatic cord dissolution, and hepatic sinusoid dilatation at 8 h pi (scale bar = 50 μm, × 40 magnification). (D) C3 protein expression in further necrosis (NE) of hepatocytes at 12 h pi (scale bar = 100 μm, × 20 magnification). (E1, E2) C3 protein expression in serious necrosis (NE) and hyperemia (excessive blood cells (BC)) of liver at 24 h pi (scale bar = 50 μm, × 40 magnification). (F) C3 protein expression in alleviated hyperemia and necrosis (NE) at 48 h pi (scale bar = 100 μm, × 20 magnification). (G) C3 protein expression in blood vessel (BV) with hyperemia, and some necrosis (NE) of hepatocytes at 72 h pi (scale bar = 100 μm, × 20 magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

control (Fig. 2A). This may suggest that the liver is not only the primary organ for synthesizing C3, but also for regulating the amount of C3 in the body. The results of C3 protein concentration showed that the peaks of C3 concentration were reached after 24 h pi (Fig. 3). The large amount of C3 could generates an excess of C3a (the anaphylactic peptide) which can mediate a multitude of functions, including the recruitment and activation of circulating macrophages and effector cells, leading to inflammatory responses [45,46]. Therefore, the liver was downregulated the C3 transcription levels to reduce the production of C3a to prevent excessive inflammatory responses from damaging self-cells.

In order to further understand the response of C3 in grass carp infected with *A. hydrophila*, we examined the intrahepatic or serous C3 protein concentrations. During bacterial infection, the liver and serum C3 protein concentrations decreased or increased to varying degrees (Fig. 3). First, the activation of the complement pathway was largely consumed the C3 protein, and then the *gcC3* transcription levels were significantly upregulated to assist C3 synthesis ($P < 0.05$; Fig. 2). Similarly, C3 protein concentrations had generally peaked after 24 h pi, the *gcC3* transcription levels were maintained at below-normal or normal levels (Figs. 2 and 3). In addition, we found the serous C3 protein concentrations decreased in a less obvious manner and soon recovered to normal levels compared to the intrahepatic C3 protein concentrations (Fig. 3). Li et al. have indicated that the number of macrophages increases significantly when grass carp infected with *A. hydrophila* [47], meanwhile Fischer et al. have indicated that macrophages are the main source of extrahepatic complement production [48], which are caused serous C3 protein levels fluctuate more smoothly.

To further understanding of the potential role of C3 protein, we explored the relationship between C3 protein level changes and tissue damage in grass carp infected with *A. hydrophila*. In the current study, we used the same semi-quantitative system as before to evaluate the health status of the liver tissue following *A. hydrophila* challenge [49].

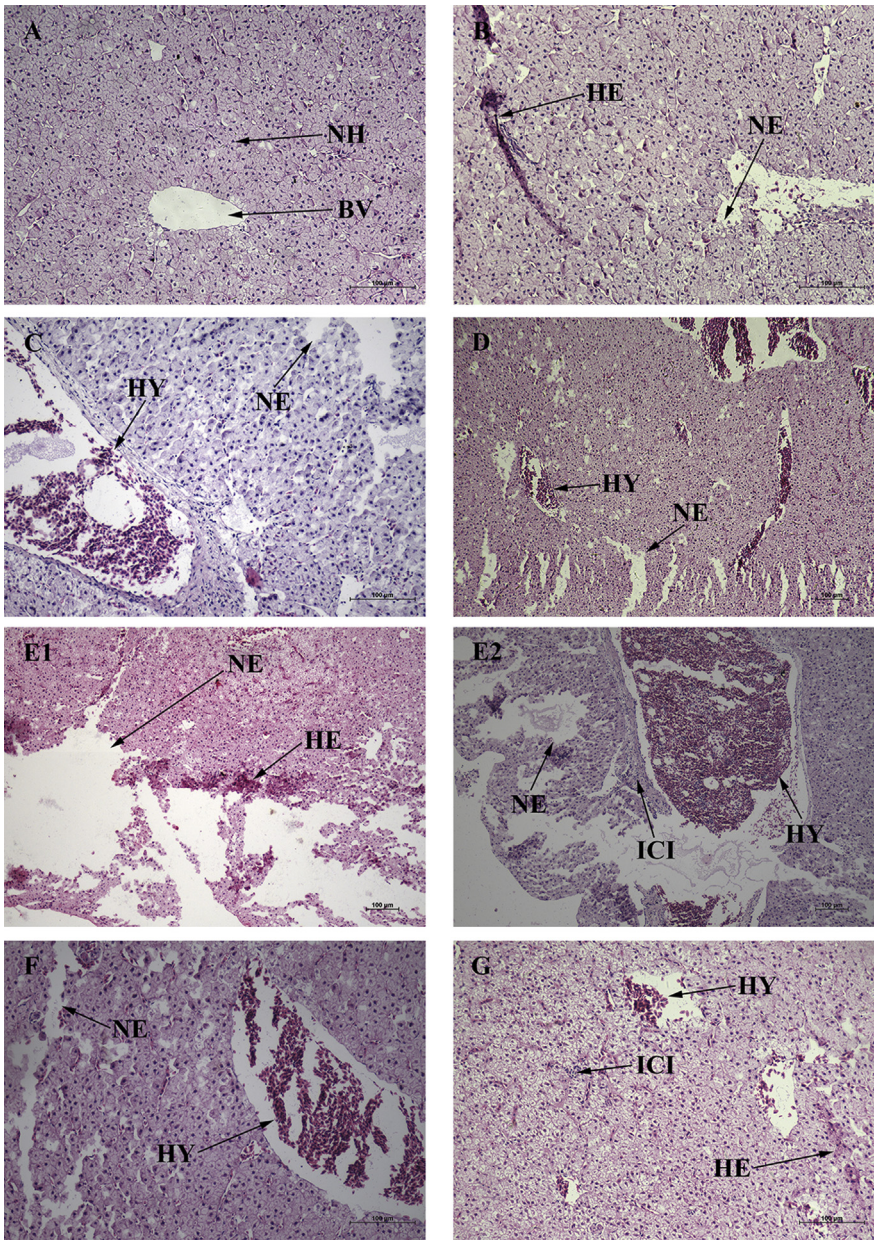

Fig. 5. Representative photos of the histological alterations in the liver throughout the experiment duration (scale bar = 100 μm). (A) Normal structure with systematic arrangement of hepatocytes (NH) and normal blood vessels (BV) (× 20 magnification). (B) Slight hemorrhage (HE) and necrosis (NE) at 4 h pi (× 20 magnification). (C) Partial necrosis (NE) and some hyperemia (HY) at 8 h pi (× 20 magnification). (D) Moderate hyperemia (HY) and further necrosis (NE) at 12 h pi; the partial hepatocytic wall disappeared and hepatocytes became irregular in shape (× 10 magnification). (E1, E2) Serious hemorrhage (HE), area of necrosis (NE), and inflammatory cell infiltration (ICI) at 24 h pi; connections between the vessels and hepatocytes were completely fractured (× 10 magnification). (F) Obvious decrease in necrosis (NE) and hyperemia (HY) at 48 h pi (× 20 magnification). (G) Some hemorrhage (HE), hyperemia (HY), and inflammatory cell infiltration (ICI) at 72 h pi (× 20 magnification).

The semi-quantitative system has also been used in many studies because it enables standardized quantification for histological analysis [32]. In our results, the liver tissues showed varying degrees of damage at the different time points (Fig. 5). Such damage can induce hydrolase overflow and cell autolysis or other inflammatory reactions [50,51], which disrupt the balance of immune status of the fish and promote complement pathway activation. By analyzing, we found that the infected fish had a significantly higher index of alterations involving necrosis and architectural or structural alterations compared to the control ($P = 0.007$; Table 3), but the C3 protein levels were not significantly downregulated at 24 h pi ($P > 0.05$; Fig. 3). The above phenomenon can be explained by previous researches of Strey et al. and Laufer et al.. Strey et al. observed systemic compensatory responses in patients with liver resection (LR), including complement activation and the release of anaphylaxis mediators [52]. And Laufer et al. indicated that extrahepatic synthesis of complement components has been well-documented in many cell types [16]. Therefore, serious liver tissue damage does not affect C3 protein levels in grass carp, systemic compensatory responses will produce different levels of C3 protein to maintain its function. After 24 h pi, the health status of the liver tissue was better than that at 24 h pi (Table 3), while liver or serum C3 concentrations were maintained at high levels compared with the control ($P < 0.05$; Fig. 3). This phenomenon demonstrated that C3 has other positive effect in grass carp after bacterial infection. Higher C3 level could help grass carp better cope with secondary infections of bacteria, allowing them to survive. Moreover, higher C3 protein concentrations may be associated with other life activities after *A. hydrophila* infection. Previous studies have indicated that complement not only participates in the immune function of body but also in other important life activities [53,54], such as tissue and organ regeneration [55–58]. From the results of immunohistochemistry (Fig. 4), C3 protein was translated near the nucleus of hepatocytes and transported into the interval of liver necrosis during infection, which may partly imply that C3 protein is involved in liver tissue repair. Clark et al. indicated that C3 is required for a normal hepatic regenerative response, but that disruption of the classical or lectin-dependent pathways (C4-dependent), the alternative pathway (factor B-dependent), or all three pathways does not impair the hepatic regenerative response, and indicated

Table 3
Liver index (LI) and Classification.

Statistical analysis	Liver index (LI)			Classification
	Observed range	Mean ± SD	Kruskal–Wallis ANOVA	
Control groups	0–2	0.7 ± 1.15		Class I
4h pi	8–12	9.3 ± 2.31	$P = 1.000$	Class I
8h pi	14–26	21.3 ± 6.43	$P = 0.363$	Class III
12h pi	18–30	22.7 ± 6.43	$P = 0.363$	Class III
24h pi	36–44	40.0 ± 4.00	$P = 0.007$	Class V
48h pi	18–22	20.7 ± 2.31	$P = 0.515$	Class III
72h pi	8–18	18.0 ± 12.49	$P = 1.000$	Class II

The LI is equal to the sum of multiple histological reaction index (IC, index for circulatory disturbances; IR, index for regressive changes; IP, index for progressive changes; II, index for inflammation; IT, index for tumor). Classification is based on the LI and includes five grades (I–V). Significant differences ($P < 0.05$) among the LI for each day were analyzed using a Kruskal–Wallis non-parametric test, followed by multiple comparisons. The *P*-value represents the result of comparisons between the experimental and control groups.

that non-traditional mechanisms by which C3 is activated during hepatic regeneration must exist [59]. In addition, some researchers have noted that serum C3 levels from male subjects without previous ischemic events are independently associated with risk of myocardial infarction [60,61]. Therefore, we made a bold prediction that the status of disease in fish could be understood by measuring the serum C3 levels in the future.

In conclusion, C3 plays an important role in fighting *A. hydrophila* infection in grass carp. Higher C3 protein concentrations could help grass carp better resist bacterial invasion and protect tissues from bacterial damage. Moreover, C3 protein plays an active repair role in injured tissue after *A. hydrophila* infection.

Acknowledgments

We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript. This work was supported by the China's Agricultural Research System [grant number CARS-45-03].

References

[1] Y.B. Shen, J.B. Zhang, J.J. Fu, X.Y. Xu, J.L. Li, R.Q. Wang, Y.F. Xuan, A 425 T > C polymorphism in complement C7 association with resistance to *Aeromonas hydrophila* in grass carp, Genet. Mol. Res. 15 (2016) 15014526.
[2] M.Y. Hu, Y.B. Shen, X.Y. Xu, H.Y. Yu, M. Zhang, Y.F. Dang, J.L. Li, Identification, characterization and immunological analysis of Ras related C3 botulinum toxin substrate 1 (Rac1) from grass carp *Ctenopharyngodon idella*, Dev. Comp. Immunol. 54 (2016) 20–31.
[3] FAO, Fisheries and Aquaculture Department [online], [Cited 30 December 2018] http://www.fao.org/fishery/culturedspecies/Ctenopharyngodon_idellus/en.
[4] Y. Dang, X. Xu, Y. Shen, M. Hu, M. Zhang, L. Li, L. Lv, J. Li, Transcriptome analysis of the innate immunity-related complement system in spleen tissue of *Ctenopharyngodon idella* infected with *Aeromonas hydrophila*, PLoS One 11 (2016) e0157413.
[5] X.Y. Xu, Y.B. Shen, J.J. Fu, F. Liu, S.Z. Guo, J.L. Li, Characterization of MMP-9 gene from grass carp (*Ctenopharyngodon idella*): an Aeromonas hydrophila-inducible factor in grass carp immune system, Fish Shellfish Immunol. 35 (2013) 801–807.
[6] H. Mch, J.D. Lambris, The complement system in teleosts, Fish Shellfish Immunol. 12 (2002) 399–420.
[7] J. Bo, J.P. Giesy, R. Ye, K.J. Wang, J.S. Lee, D.W. Au, Identification of differentially expressed genes and quantitative expression of complement genes in the liver of marine medaka *Oryzias melastigma* challenged with *Vibrio parahaemolyticus*, Comp. Biochem. Physiol. Genom. Proteonom. 7 (2012) 191–200.
[8] W.E. Paul, Fundamental Immunology, seventh ed., Lippincott Williams & Wilkins, UAS, 2013.
[9] M.C. Carroll, The complement system in regulation of adaptive immunity, Nat. Immunol. 5 (2004) 981–986.
[10] M. Ruslan, J. Charles A, Decoding the patterns of self and nonself by the innate immune system, Science 296 (2002) 298–300.
[11] D. Ricklin, G. Hajishengallis, K. Yang, J.D. Lambris, Complement: a key system for immune surveillance and homeostasis, Nat. Immunol. 11 (2010) 785–797.
[12] P.F. Zipfel, W. Reinhard, S. Christine, Complement evasion of pathogens: common strategies are shared by diverse organisms, Mol. Immunol. 44 (2007) 3850–3857.
[13] R.K. Del, P.A. Tsonis, I.K. Zarkadis, A.G. Tsagas, J.D. Lambris, Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles, J. Immunol. 35 (1998) 6819–6824.
[14] R. Reca, D. Mastellos, M. Majka, L. Marquez, J. Ratajczak, S. Franchini, A. Glodek, M. Honczarenko, L.A. Spruce, A. Janowska-Wieczorek, Functional receptor for C3a anaphylatoxin is expressed by normal hematopoietic stem/progenitor cells, and C3a enhances their homing-related responses to SDF-1, Blood 101 (2003) 3784–3793.
[15] L. Kouser, M. Abdul-Aziz, A. Nayak, C.M. Stover, R.B. Sim, U. Kishore, Properdin and factor H: opposing players on the alternative complement pathway "See-Saw", Front. Immunol. 4 (2013) 93.
[16] J. Laufer, Y. Katz, J.H. Passwell, Extrahepatic synthesis of complement proteins in inflammation, Mol. Immunol. 38 (2001) 221–229.
[17] M. Løvoll, T. Kilvik, H. Boshra, J. Bøgwald, J.O. Sunyer, R.A. Dalmo, Maternal transfer of complement components C3-1, C3-3, C3-4, C4, C5, C7, Bf, and Df to offspring in rainbow trout (*Oncorhynchus mykiss*), Immunogenetics 58 (2006) 168–179.
[18] S. Lange, S.H. Bambir, A.W. Dodds, T. Bowden, I. Bricknell, S. Espelid, B. Magnadóttir, Complement component C3 transcription in Atlantic halibut (*Hippoglossus hippoglossus L.*) larvae, Fish Shellfish Immunol. 20 (2006) 285–294.
[19] S. Lange, A.W. Dodds, S. Gudmundsdóttir, S.H. Bambir, B. Magnadóttir, The ontogenic transcription of complement component C3 and Apolipoprotein A-I tRNA in Atlantic cod (*Gadus morhua L.*)—a role in development and homeostasis? Dev. Comp. Immunol. 29 (2005) 1065–1077.
[20] B. Magnadottir, S. Lange, S. Gudmundsdottir, J. Bøgwald, R.A. Dalmo, Ontogeny of humoral immune parameters in fish, Fish Shellfish Immunol. 19 (2005) 429–439.
[21] A.S. Giasuddin, J.M. Elmahdawi, F.M. Elhassadi, Serum complement (C3, C4) levels in patients with acute myocardial infarction and angina pectoris, Bangladesh Med. Res. Counc. Bull. 33 (2007) 98–102.
[22] T. Monowar, M. Rahman, O. Chowdhury, M. Shahabuddin, A. Kundu, Serum complement (C3, C4) levels in patients with acute myocardial infarction, Restaur. Des. 2 (2013) 74–89.
[23] M.M. Markiewski, D. Mastellos, R. Tudoran, R.A. Deangelis, C.W. Strey, C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury, J. Immunol. 173 (2018) 747–754.
[24] K. Pushpa, P. Gireesh-Babu, K.V. Rajendran, C.S. Purushothaman, S. Dasgupta, M. Makesh, Molecular cloning, sequencing and tissue-level expression of complement C3 of Labeo rohita (Hamilton, 1822), Fish Shellfish Immunol. 40 (2014) 319–330.
[25] H. Wang, P. Qi, B. Guo, J. Li, J. He, C. Wu, Y. Gul, Molecular characterization and expression analysis of a complement component C3 in large yellow croaker (*Larimichthys crocea*), Fish Shellfish Immunol. 42 (2015) 272–279.
[26] M. Chang, P. Nie, The cloning and expression of the complement C3 gene in grass carp infected *Sinergasilus major*, Prog. Nat. Sci. 14 (2004) 870–881 (in Chinese).
[27] X. Xu, Y. Shen, J. Fu, L. Lu, J. Li, Next-generation sequencing identified microRNAs that associate with motile aeromonad septicemia in grass carp, Fish Shellfish Immunol. 45 (2015) 94–103.
[28] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods 25 (2001) 402–408.
[29] L. Xiaoyu, L. Li, Z. Yaning, F. Qian, L. Yuanyuan, L. Yuanlong, Toxic effects of chlorpyrifos on lysozyme activities, the contents of complement C3 and IgM, and IgM and complement C3 expressions in common carp (*Cyprinus carpio L.*), Chemosphere 93 (2013) 428–433.
[30] G.L. Humason, Animal Tissue Techniques, fourth ed., Freeman and Company, New York, 1979.
[31] J.C.V. Dyk, G.M. Pieterse, A histo-morphological study of the testis of the sharp-tooth catfish (*Clarias gariepinus*) as reference for future toxicological assessments, J. Appl. Ichthyol. 24 (2008) 415–422.
[32] D. Bernet, H. Schmidt, W. Meier, P. Burkhardt-Holm, T. Wahli, Histopathology in fish: proposal for a protocol to assess aquatic pollution, J. Fish. Dis. 22 (1999) 25–34.
[33] S. Zimmerli, D. Bernet, P. Burkhardt-Holm, H. Schmidt-Posthaus, P. Vonlanthen, T. Wahli, H. Segner, Assessment of fish health status in four Swiss rivers showing a decline of brown trout catches, Aquat. Sci. 69 (2007) 11–25.
[34] A. Sahu, J.O. Sunyer, W.T. Moore, M.R. Sarrias, A.M. Soulika, J.D. Lambris, Structure, functions, and evolution of the third complement component and viral molecular mimicry, Immunol. Res. 17 (1998) 109–121.
[35] Y. Dang, X. Meng, S. Wang, L. Li, M. Zhang, M. Hu, X. Xu, Y. Shen, L. Lv, R. Wang, Mannose-binding lectin and its roles in immune responses in grass carp (*Ctenopharyngodon idella*) against *Aeromonas hydrophila*, Fish Shellfish Immunol. 72 (2018) 367–376.
[36] Y. Dang, Y. Shen, X. Xu, S. Wang, X. Meng, M. Zhang, L. Lv, R. Wang, J. Li, Complement component Bf/C2b gene mediates immune responses against *Aeromonas hydrophila* in grass carp *Ctenopharyngodon idella*, Fish Shellfish Immunol. 74 (2018) 509–516.
[37] Y. Shen, J. Zhang, X. Xu, J. Fu, J. Li, Expression of complement component C7 and involvement in innate immune responses to bacteria in grass carp, Fish Shellfish Immunol. 33 (2012) 448–454.
[38] S.R. Barnum, Complement biosynthesis in the central nervous system, Crit. Rev. Oral Biol. Med. 6 (1995) 132–146.
[39] P. Gasque, Y.D. Dean, E.P. Mcgreal, J. Vanbeek, B.P. Morgan, Complement components of the innate immune system in health and disease in the CNS, Immunopharmacology 49 (2000) 171–186.
[40] M. Nakao, J. Mutsuro, R. Obo, K. Fujiki, M. Nonaka, T. Yano, Molecular cloning and

protein analysis of divergent forms of the complement component C3 from a bony fish, the common carp (*Cyprinus carpio*): presence of variants lacking the catalytic histidine, Eur. J. Immunol. 30 (2000) 858–866.
[41] J. Sigh, T. Lindenstrøm, K. Buchmann, The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish. Dis. 27 (2004) 409–417.
[42] Z.H. Qi, Y.F. Liu, W.N. Wang, X. Wu, Y. Xin, Y.F. Lu, A.L. Wang, Molecular characterization and functional analysis of a complement C3 molecule in the orange-spotted grouper (*Epinephelus coioides*), Fish Shellfish Immunol. 31 (2011) 1284-1920.
[43] J.P. Saeij, B.J. de Vries, G.F. Wiegertjes, The immune response of carp to *Trypanoplasma borreli*: kinetics of immune gene expression and polyclonal lymphocyte activation, Dev. Comp. Immunol. 27 (2003) 859–874.
[44] I. Mauri, N. Roher, S. Mackenzie, A. Romero, M. Manchado, J.C. Balasch, J. Béjar, M.C. Alvarez, L. Tort, Molecular cloning and characterization of European seabass (*Dicentrarchus labrax*) and Gilthead seabream (*Sparus aurata*) complement component C3, Fish Shellfish Immunol. 30 (2011) 1310–1322.
[45] E.A. Nordahl, V. Rydengard, P. Nyberg, D.P. Nitsche, M. Mörgelin, M. Malmsten, L. Björck, A. Schmidtchen, Activation of the complement system generates antibacterial peptides, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 16879–16884.
[46] W. Zhou, The new face of anaphylatoxins in immune regulation, Immunobiology 217 (2012) 225–234.
[47] L. Li, Y. Dang, Y. Shen, X. Xu, W. Huang, J. Li, Hematological and Immunological plasma assays for grass carp (*Ctenopharyngodon idella*) infected with *Aeromonas hydrophila* as a immune model in carp aquaculture, Fish Shellfish Immunol. 55 (2016) 647–653.
[48] M.B. Fischer, M. Ma, N.C. Hsu, M.C. Carroll, Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response in C3-deficient mice, J. Immunol. 160 (1998) 2619–2625.
[49] S.T. Wang, X.Z. Meng, L.S. Li, Y.F. Dang, Y. Fang, Y. Shen, X.Y. Xu, R.Q. Wang, J.L. Li, Biological parameters, immune enzymes, and histological alterations in the livers of grass carp infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 70 (2017) 121–128.
[50] C.Y. Wang, The pathogenicity and drug sensitivity tests of bacterial septicemia from *C.giblio*, J. Biol. 19 (2002) 30–31.
[51] M. Yang, Prevention and cure on infection of hemorrhagic septicemia of Mud Eel, J. Xiaogan Univ. (Nat. Sci.) 18 (1998) 70–74.
[52] C.W. Strey, B. Siegmund, S. Rosenblum, R.M. Marquez-Pinilla, E. Oppermann, M. Huber-Lang, J.D. Lambris, W.O. Bechstein, Complement and neutrophil function changes after liver resection in humans, World J. Surg. 33 (2009) 2635–2643.
[53] M. Huber-Lang, A. Kovtun, A. Ignatius, The role of complement in trauma and fracture healing, Semin. Immunol. 25 (2013) 73–78.
[54] A.H. Stephan, B.A. Barres, B. Stevens, The complement system: an unexpected role in synaptic pruning during development and disease, Annu. Rev. Neurosci. 35 (2012) 369–389.
[55] P.S. Heeger, C. Kemper, Novel roles of complement in T effector cell regulation, Immunobiology 217 (2012) 216–224.
[56] D. Mastellos, C. Andronis, A. Persidis, J.D. Lambris, Novel biological networks modulated by complement, Clin. Immunol. 115 (2005) 225–235.
[57] D. Mastellos, J.D. Lambris, Complement: more than a 'guard' against invading pathogens? Trends Immunol. 23 (2002) 485–491.
[58] D. Mastellos, D. Morikis, C. Strey, M.C. Holland, J.D. Lambris, From atoms to systems: a cross-disciplinary approach to complement-mediated functions, Mol. Immunol. 41 (2004) 153–164.
[59] A. Clark, A. Weymann, E. Hartman, Y. Turmelle, M. Carroll, J.M. Thurman, V.M. Holers, D.E. Hourcade, D.A. Rudnick, Evidence for non-traditional activation of complement factor C3 during murine liver regeneration, Mol. Immunol. 45 (2008) 3125–3132.
[60] A. Muscari, C. Bozzoli, G.M. Puddu, Z. Sangiorgi, A. Dormi, C. Rovinetti, G.C. Descovich, P. Puddu, Association of serum C3 levels with the risk of myocardial infarction, Am. J. Med. 98 (1995) 357–364.
[61] A. Muscari, G. Massarelli, L. Bastagli, G. Poggiopollini, V. Tomassetti, G. Drago, C. Martignani, P. Pacilli, P. Boni, P. Puddu, Relationship of serum C3 to fasting insulin, risk factors and previous ischaemic events in middle-aged men, Eur. Heart J. 21 (2000) 1081–1090.