

Molecular Characterization of A New IgZ3 Subclass from Common Carp (*Cyprinus Carpio*) and Comparative Expression Analysis of IgH Transcripts During Ontogeny of Larvae

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Abstract

Background Immunoglobulins distributing among systemic immune tissues and mucosal immune tissues play an important role in teleost to protect them from infections in a pathogen-rich aquatic environment. Teleost IgZ/IgT subclasses with different tissue expression patterns may have varied immune functions.

Results In the present study, a novel secreted IgZ heavy chain gene was cloned and characterized in common carp (*Cyprinus carpio*) which was different from the reported IgZ1 and IgZ2 in tissue-specific expression profile. The obtained IgZ-like subclass was designated as *CdgZ3* which complete open reading frame contained 1650 bp encoding a protein of 549 amino acid residues. The phylogenetic analysis revealed that *CdgZ3* was grouped with carp IgZ2 and was in the same branch as other teleosts IgZ/IgT. Basal expression detection of IgH in healthy adult common carp showed that *CdgZ3* transcripts were widely expressed in systemic immune tissues and Mucosal-associated lymphoid tissues. It was expressed at a higher level in the head kidney, gill, and gonad, followed by spleen, hindgut, oral epithelium, liver, brain, muscle, foregut, and blood, but at a very low level in the skin. The transcript level of *CdgZ3* mRNA in isolated leukocytes from peripheral blood cells was significantly higher than that of isolated leukocytes from the spleen. Different groups of common carp were infected with *Aeromonas hydrophila* via intraperitoneal injection and immersion respectively. The qRT-PCR analysis demonstrated that a significant difference in *CdgZ3* mRNA level between immersion and injection groups existed in all the detected tissues including head kidney, spleen, liver, and hindgut, especially in hindgut *CdgZ3* mRNA level was higher in immersion than in the injection group. Different routes for *Aeromonas hydrophila* challenge to common carp had comparatively less effect on IgM response. Further study on the relative expression of the IgH gene during the ontogeny of common carp presented that the tissue-specific expression profile of *CdgZ3* was very different from the others. In the early larval development stage of common carp from 1 dpf to 31 dpf, the qRT-PCR analysis demonstrated that the *CdgZ3* mRNA level increased gradually with a similar dynamic tendency of IgZ1 and IgZ2 and IgM was the dominant Ig with obviously higher abundance. The results of tissue-specific expression of IgH at 65 dpf of common carp showed that *CdgZ3* was expressed at mucosal sites including both hindgut and gill but IgZ1 was preferentially expressed in hindgut and IgZ2 in gill. Except for qRT-PCR analysis, the detection of the *CdgZ3*-expressing cells and IgM-expressing cells by *in situ* hybridization was performed. The results showed that *CdgZ3* and IgM transcripts could be detected in the spleen, gill, and hindgut of the common carp at 65dpf.

Conclusions These results revealed that *CdgZ3* gene transcript occurred in the early development stage of common carp not only in systemic tissues but also in mucosal tissues. *CdgZ3* could be induced with significantly different expression profiles at immune tissues after the challenge by *Aeromonas hydrophila* immersion and intraperitoneal injection, which indicated that *CdgZ3* might play a more important role in mucosal immunity than in systemic immunity.

Background

Fish, like other vertebrates, possess an extensive defense system, which enables the individual to survive and maintain its integrity in a hostile environment. The humoral immune system responds to a variety of pathogens by producing specific antibodies. Antibodies produced by B lymphocytes were existed in vertebrates from mammals to cartilaginous fish[1]. There are five types of heavy chains in mammals, including μ , δ , γ , ϵ , and κ , which differ in their constant region. Antibodies that contain different heavy chains belong to different isotypes and are named IgM, IgD, IgG, IgE, and IgA respectively. Each isotype has distinct physical and biological properties and effector functions. Fish are the most primitive group of vertebrates that possess an adaptive immune system capable of generating antibodies in response to pathogenic challenges. Except for IgM and IgD, a novel isotype IgZ(ζ) or IgT (τ) was identified in many teleost fish species[2], which were also found to have varied subclasses with different gene sequence and different tissue expression patterns, Such as IgZ1 and IgZ2 in zebrafish (*Danio rerio*)[3], IgT1, IgT2, IgT3, IgT4, and IgT5 in rainbow trout (*Oncorhynchus mykiss*)[4], IgZ and a chimeric IgZ (IgZ2) in grass carp (*Ctenopharyngodon idellus*)[5], IgT1, IgT2, IgT3 and IgT4 in stickleback (*Gasterosteus aculeatus*)[6], five IgT-A and three IgT-B in Atlantic salmon (*Salmo salar*) [7]and IgZ1 and IgM-IgZ (IgZ2) in common carp (*Cyprinus carpio*)[8]. Most of the IgZ/T molecules have four CH domains which were encoded by the C ζ /C τ genes, but some others have two CH domains or three CH domains, such as two constant domains in common carp (*Cyprinus carpio*) IgZ2 [9]and torafugu (*Takifugu rubripes*) IgH[10], three constant domains in stickleback (*Gasterosteus aculeatus*) IgT[6] and sea bass (*Dicentrarchus labrax*)[11]. Except for the diversity of the domain number of IgZ/T subclasses among fish species, those IgZ/T subclasses displayed varied expression patterns and functions. The reported IgZ1 of common carp is mainly expressed in blood against blood pathogens, and IgZ2 chimera is preferentially expressed in the mucosal compartment to respond to mucosal infections[8]. The rainbow trout Ig τ 1 was mainly expressed in both systemic and mucosal lymphoid tissues, while Ig τ 2 was largely expressed in systemic lymphoid organs. After poly (I:C) treatment, Ig τ 1 and Ig τ 2 genes exhibited different expression profiles, Ig τ 1 transcripts reached a peak at 7 d in the spleen and 14 d in the gut, However, Ig τ 2 increased slightly and reached a peak at 7 d in both the spleen and gut[12]. The previous study about this novel IgH indicated that teleost fish IgZ/T was more diverse than previously thought.

Teleost IgZ/T was considered as a primitive immunoglobulin class specialized in mucosal immunity, which is equivalent to immunoglobulin IgA in mammals and plays an important role in the mucosal immune response[2, 13]. IgZ/T is a comparatively new teleost immunoglobulin class and its presence, expression, and tissue distribution during the early development stage are not very clear. Most studies have been investigated on the ontogeny of IgM positive cells and IgM-secreting cells. The previous studies indicated that the first B cells are most probably generated in the head kidney and B cells populate the gut-associated lymphoid tissue (GALT) much later than the spleen or kidney[14]. In channel catfish, specific IgM in eggs could be detected, which provides an immune barrier at the surface of the egg, as well as protection for the developing fry. In carp, surface IgM⁺ cells were first detected in the head kidney at 2 weeks post-fertilization using WCI12 and WCI4, monoclonal antibodies against IgM H chain, and carp injected with T cell-independent antigen (LPS) developed antibody responses and memory from 4 wpf, while they responded against T cell-dependent antigens (HGG) from 8 wpf[15]. By using specific

primers designed according to the IgH constant sequence, qRT-PCR was performed to detect the IgH presence during the early development stage of some teleost fish species. In zebrafish, all the Ig isotypes effectively responded to the LPS challenge from 21 dpf onwards, IgZ1 responded to LPS challenge faster and stronger than IgM and IgD on 28 dpf. IgZ-2 transcripts could be detected at 14 days after fertilization[16]. In common carp whole embryos showed constitutive expression of all three immunoglobulins (IgM, IgZ1, and IgZ2) as early as 4 dpf with IgM being the predominant form. IgZ1 and IgZ2 expression increased rapidly to reach their maximum at 12 dpf whereas IgM expression reached its maximum at 30 dpf[8]. The appearance of immunoglobulin during the early development stage varies considerably in different teleost species due to important differences in developmental status at hatch and the aquatic environment. The presence of Ig molecules in fish embryos and larva suggests their role as an important barrier against pathogens.

Carp is one of the most popular cultured fish in China and the diseases caused by *Aeromonas hydrophila* can do great harm to carp culture. *Aeromonas hydrophila* is resistant to antibiotics attributed to the indiscriminate use of antibiotics in aquaculture. Vaccines based on the immune response mediated by antibodies will help to defend against bacterial infection without the use of antibiotics[17]. In the present study, we cloned a new *CclgZ3* gene from common carp and detected its expression pattern in the adults and during embryonic development of common carp with comparison to the other known IgH of this species. The immune response of *CclgZ3* and IgM after the challenge by *Aeromonas hydrophila* by immersion and injection were also analyzed comparatively. Our results provide additional experimental evidence to understand the IgZ/T ontogeny and immune function in teleost fish.

Results

Molecular cloning and analysis of common carp *CclgZ3*

Identification of the constant region of CclgZ3 cDNA in Cyprinus carpio.

The obtained sequence of full-length *CclgZ3* cDNA was 2144 bp with a 5'-UTR of 112 bp and a 3'-UTR of 382 bp with a putative typical polyadenylation signal sequence AATAAA located upstream of the poly (A) tail. The deduced *CclgZ3* amino acid sequence contained 549 aa and spanned V domain, four constant domains and a secreted tail (Fig. 1.). The analysis of the IgT sequence showed the presence of a putative 20 amino acids signal peptide. Concerning the V domain of the *CclgZ3* sequences the IMGT unique numbering has been used. The entire C-domain could be divided into four CH domains.

Multiple amino acid sequence alignment

The alignment of common carp *CclgZ3* with zebrafish IgZ1 and IgZ2, common carp IgZ1 and IgZ2, grass carp IgZ and IgZ2 showed that it was comprised of four Ig-like constant domains (CH1, CH2, CH3, CH4) and a secretory tail. The conserved cysteine residues for disulfide bond formation and tryptophan

residues for the folding of IgSF domain were present in each CH domain of *CclgZ3* (Fig. 2.). Five N-linked glycosylation sites were predicted to be present in *CclgZ3* (in CH2, CH3 and CH4) (Fig. 1.). The number and distribution of putative N-glycosylation sites including the sequons NXS or NXT for each domain are varied in different teleost species. CH1 domain could possess none to three putative N-glycosylation sites. There was no N-linked glycosylation site in *CclgZ3* CH1 domain, which is different from the grass carp (three N-linked glycosylation sites in CH1) and zebrafish (two N-linked glycosylation sites in CH1). The first N-linked glycosylation site of *CclgZ3* existed in the CH2 domain and it is at the very beginning of CH2. *CclgZ3* presented two N-linked glycosylation sites in CH3 and CH4 domains respectively, which is similar to the grass carp[5].

The percentage of identity between the amino acid sequences of all the IgZ subclasses of common carp and that of grass carp, zebrafish and rainbow trout were calculated considering every single domain. The results showed that the value of identity obtained ranged between 37.5% and 74.76% for all the three IgZ subclasses of common carp, 11.59% and 70.21% for the three IgZ subclasses of common carp with grass carp IgZ/IgZ2, 24.18% and 64.13% for the three IgZ subclasses of common carp with zebrafish IgZ1/IgZ2, 21% and 40.82% for the three IgZ subclasses of common carp with rainbow trout IgT1 ~ IgT3. Interestingly, the identity between *CclgZ3* CH1 domain and grass carp IgZ CH2 domain (71.26%) was higher than that between *CclgZ3* CH1 domain and grass carp IgZ CH1 domain (11.69%), the other two IgZ of common carp also showed this characteristic (41.67% vs 13.1 in IgZ1, 66.67% vs 17.07% in IgZ2).

Phylogenetic analysis

The phylogenetic analysis of immunoglobulins in vertebrates was performed to reveal the relationship of common carp *CclgZ3* with other immunoglobulins. The amino acid sequences from different species including mammals, birds, amphibians and fish were aligned by Clustal X2 program. Based on the alignment, we constructed an NJ tree supported by 1000 bootstrap replications. It showed that common carp *CclgZ3* are grouped with grass carp IgZ and in the same branch as IgZ/T, further demonstrated that *CclgZ3* is a homology of IgZ (Fig. 3).

Basal expression comparison of *CclgZ3* with other Ig molecules of common carp

To investigate the tissue-dependent expression pattern, we performed qRT-PCR analysis using gene-specific primers for all the three IgZ and IgM of common carp in normal adult common carp tissues including liver, spleen, head kidney, gill, skin, gonad, brain, muscle, blood, foregut, hindgut and oral epithelium. The expression of the *CclgZ3*, IgZ1, IgZ2 and IgM were found in all the tested tissues and IgM was the most abundant Ig in all the tissues. The expression of *CclgZ3* was found to be highest in the head kidney, gill, and gonad, followed by spleen, hindgut, oral epithelium, liver, brain, muscle, foregut, and blood, but at a very low level in skin. IgZ1 and IgZ2 demonstrated similar expression patterns with *CclgZ3* in most of the detected tissues except that the lowest transcription level of IgZ1 and IgZ2 were found in blood (Fig. 4). To compare the expression of the four IgH transcripts in leukocytes, we performed qRT-PCR analysis using gene-specific primers for all the three IgZ subclasses and IgM of common carp in isolated

leukocytes from peripheral blood cells and spleen. The result showed that significant difference in *CdIgZ3* expression existed between PBL and leukocytes from the spleen. In contrast, no significant difference in IgM, IgZ1 and IgZ2 expression existed between PBL and leukocytes of the spleen (Fig. 5).

Basal expression comparison of CcIgZ3 with other Ig molecules of common carp during the different developmental stage

When testing for constitutive expression of the four immunoglobulin genes during the early development stage of common carp by qRT-PCR, expression was recorded from 1 to 65 days post fertilization (dpf). (Fig. 6 and Fig. 7). Expression of *CdIgZ3* and the other three immunoglobulin genes were detected from 6 days post fertilization (dpf). IgM was expressed highest and was the dominant immunoglobulin isotype during the early development stages tested. (Fig. 6). Furthermore, to investigate the tissue expression pattern of the IgH transcripts, tissues including gill, spleen, hindgut and liver from common carp at 65 days post fertilization (dpf) were used to evaluate the expression level of *CdIgZ3*, IgM, IgZ1, and IgZ2 respectively. The expression of IgM was still higher than that of the other three subclasses and it was the only isotype that could be detected in all the tested tissues. The gene expression of *CdIgZ3*, IgZ1 and IgZ2 exhibited varied tissue expression preference. *CdIgZ3* was found in spleen, hindgut and gill but not in liver, however, IgZ1 was expressed at a higher level in liver but its expression level in gill was too low to be detected. IgZ2 was primarily detected in gill and spleen but hardly detected in liver and hindgut (Fig. 7).

Tissue location of CcIgZ3 and IgM mRNA-expressing cells at early development stage of common carp

As both of the *CdIgZ3* and IgM expression were detected in spleen, gill and hindgut from common carp at 65 dpf, we further detected the *CdIgZ3* mRNA-expressing cells and IgM mRNA-expressing cells in tissues by *in situ* hybridization. Positive expression of both IgM and *CdIgZ3* were seen in splenocytes (Fig. 8F and 8H), gill epithelial cells (Fig. 8J and 8L) and cells in lamina propria of hindgut (Fig. 8B and 8D). The staining was specific for both IgM and *CdIgZ3* as only the antisense probes (Fig. 8B, 8D, 8F, 8H, 8J, 8L) and not the sense probes (Fig. 8A, 8C, 8E, 8G, 8I, 8K.) hybridized.

Organ-specific CcIgZ3 and IgM expression after *Aeromonas hydrophila* challenge by different routes

The qRT-PCR results showed that both *CdIgZ3* and IgM mRNA expressions were up-regulated compared to the blank control in all tested tissues in the injection and immersion group from 3 days to 21 days except for *CdIgZ3* in liver in injection group (0.46-fold at 3 days) (Fig. 9F). Compared with the control, the peak value of *CdIgZ3* expression was 86.78-fold in spleen (Fig. 9D), 31-fold in hindgut (Fig. 9H), 13.61-fold in head kidney (Fig. 9B), and 6.39-fold in liver (Fig. 9F) in the immersion group, and 65.13-fold in spleen (Fig. 9D), 10.96-fold in hindgut (Fig. 9H), 26.91-fold in head kidney (Fig. 9B), and 1.21-fold in liver (Fig. 9F) in the injection group respectively. The peak expression of *CdIgZ3* in immersion group, in

contrast to injection group was comparatively higher in liver (5.28-fold), hindgut (2.83-fold), and spleen (1.33-fold), but lower in head kidney (0.51-fold). However, the peak value of IgM expression was 13.79-fold in head kidney (Fig. 9A), 0.57-fold in spleen (Fig. 9C), 6.5 in liver (Fig. 9E) and 4.07 in hindgut (Fig. 9G) in immersion group and 3.09-fold in head kidney (Fig. 9A), 5.27-fold in spleen (Fig. 9C), 5.85-fold in liver (Fig. 9E), and 5.8-fold in hindgut (Fig. 9G) in injection group. The peak expression of IgM in immersion group, in contrast to injection group were comparatively higher in head kidney (4.46-fold), spleen (1.25-fold), and liver (1.11-fold), but lower in hindgut (0.7-fold). The results of IgM displayed different tissue expression pattern and lower fold change from that of *CdglZ3*.

The peak time of *CdglZ3* and IgM were most at 3–7 days in spleen, liver, and hindgut in both immersion group and injection group. However, the peak time of IgM in head kidney was much later in immersion group (14 days) than in injection group (1 day). The peak time of *CdglZ3* in head kidney was also later in immersion group (14 days) than in injection group (3 days). In hindgut, the peak time of *CdglZ3* appeared later in immersion group (3 days) than in injection group (1 day). The immersion challenge in these tissues showed a slower immune response mediated by *CdglZ3* and IgM than injection challenge (Fig. 9).

As for the response in hindgut, the up-regulation of *CdglZ3* expression was significantly higher in the immersion group than the injection group from 3 days to 21 days post challenge, while the expression of IgM had no significant difference from 3 days to 21 days in the hindgut. The value of *CdglZ3* expression in hindgut was 1.73-fold at 1 day, 31-fold at 3 days, 11.53-fold at 7 days, 5.97-fold at 14 days, and 3.76-fold at 21 days in immersion group, and 10.96-fold at 1 day, 1.69-fold at 3 days, 1.13-fold at 7 days, 1.06-fold at 14 days, and 1.09-fold at 21 days in injection group. The ratio of expression of *CdglZ3* in immersion group in contrast to injection group at different time point were 0.15-fold at 1 day, 18.34-fold at 3 days, 10.2-fold at 7 days, 15.63-fold at 14 days, and 3.45-fold at 21 days (Fig. 9H). The value of IgM expression in hindgut was 0.75-fold at 1 day, 2.93-fold at 3 days, 4.07-fold at 7 days, 2.07-fold at 14 days, and 1.73-fold at 21 days in immersion group, and 2.94-fold at 1 day, 2.13-fold at 3 days, 5.80-fold at 7 days, 3-fold at 14 days, and 1.62-fold at 21 days in injection group. The ratio of expression of IgM in immersion group in contrast to injection group at different time points was 0.26-fold at 1 day, 1.38-fold at 3 days, 0.7-fold at 7 days, 0.69-fold at 14 days, and 1.07-fold at 21 days (Fig. 9G). The results showed that *Aeromonas hydrophila* challenge triggered a stronger *CdglZ3* immune response than IgM in hindgut, especially by the immersion route.

Discussion

In teleost, apart from IgM and IgD, the new IgZ/T isotype was discovered and more than one subclass of IgZ/T was found in some fish species. Although the alignment of these subclass sequence with each other indicated that every subclass of IgZ/IgT exhibited some common structural features, specific gene organization and conserved amino acid residues, their expression and distribution pattern in immune organs and tissues varied obviously, which implying the function difference among these IgZ/T molecules [18, 19].

So far in common carp, two IgZ subclasses have been reported, one is IgZ1 and the other is chimeric IgZ2, both of them have the membrane form as well as secretory form. Alignment of *CdIgZ3* with other IgZ showed that conserved cysteine residues that participate in the formation of intrachain disulfide bonds were found in each CH domain of all the three IgZ subclasses from common carp and the conserved cysteine residue for interchain disulfide bond existed in all the three IgZ CH1 domain. However, the conserved tryptophan residues for the folding of IgSF domain were just found in each CH domain of *CdIgZ3* and IgZ2 and the position of tryptophan residue in CH4 domain of *CdIgZ3* and IgZ2 was different from rainbow trout and zebrafish. The sequence of the secretory tail is conserved among the three subclasses. Alignment of the CH sequences indicated that the CH4 domain is the most highly conserved domain and CH1 domain exhibited a comparatively lower level of sequence identity. Phylogenetic analysis showed that *CdIgZ3* was grouped with IgZ2 together and the sequence identity between the two molecule CH1 and CH4 domain were 69.52% and 74.49%. In addition, the *CdIgZ3* CH1 domain was highly conserved with grass carp IgZ CH2 domain, the sequence identity of their CH1 domain was very low (11.69%). These differences made it possible to discriminate the IgZ subclasses in detecting their expression and position.

In the current study, the results of constitutive expression of different subclasses in normal tissues showed that IgZ2 chimera expressed at a slightly higher level in mucosal tissues including gill, skin, hindgut and oral epithelium compared to IgZ1 and *CdIgZ3*, however, IgZ2 expressed at a lower level in the blood than the other three subclasses, which may indicate that IgZ2 chimera was preferentially expressed at mucosal sites. This result corroborates the previous findings which found that the IgZ2 was expressed at relatively higher levels in gut and gills[8]. Compared to all the three subclasses of IgZ, IgM was predominantly expressed in all organs including both lymphoid and non-lymphoid organs and tissues. IgZ1 is expressed systemically since its comparatively higher expression level to IgZ2 and *CdIgZ3* was observed in blood. As the third member of new immunoglobulin subclasses, *CdIgZ3* expressed higher in gonad and liver compared to IgZ1 and IgZ2. To investigate whether *CdIgZ3* has the immune function, the challenged experiment was performed by *A. hydrophila* stimulation. We found that the *CdIgZ3* expression could be induced in the hindgut strongly in the challenge by *A. hydrophila* immersion, although its basal expression was not very high in hindgut. Teleost gut interacted with the foreign pathogens from water directly and was a main site for pathogens entry in teleost fish. Teleost Igs were proved to elicit the mucosal immune response in GALT by specific B lymphocytes proliferation and secreting antibodies locally within the mucosa-associated lymphoid tissues (MALTs)[20, 21]. The posterior segment of the teleost intestine was discovered to contain several immune cell types and showed to play a more important role in immune responses against pathogen invasion than the first segment and the second segment of teleost intestine[12, 21]. The *CdIgZ3* immune response in hindgut of common carp indicated that it may play an important role in protecting the host from *A. hydrophila* infection, which immune response was even stronger than that of IgM.

The ontogeny studies of fish immunoglobulin have been conducted in several species including common carp IgZ1 and IgZ2. In the previous studies, IgM and IgZ were expressed during the same stage of development in fugu [10]and trout[22], but in zebrafish[4], IgZ was the first detected antibody. In common

carp, the IgZ1 and IgZ2 could already be detected as early as 4dpf and tissue-specific expression firstly existed in the head and trunk kidney with the highest expression[8, 9]. To compare the different subclasses, we firstly performed the detection of IgM, CdgZ3, IgZ1, and IgZ2 expression during the early development stage from 1 to 31 dpf by qRT-PCR. In the current study, IgM and CdgZ3 were expressed from the same stage of 6 dpf during all the detected time points and their expression increased gradually. However, IgZ1 and IgZ2 were expressed from 16 dpf and their expression level even increased exceeding CdgZ3 at 31 dpf with a greater slope of the curve. Secondly, the results of tissue specific expression at 65 dpf showed that IgM was the only antibody that could be detected in the four tissues and it was abundant comparatively in spleen, hindgut and gill. The CdgZ3 dominantly existed in the spleen, followed by gill and hindgut, the IgZ1 gene was expressed at relatively higher levels in liver, then in hindgut and spleen, and the IgZ2 gene was expressed in gill and spleen with absence in hindgut and liver. These observations may indicate the functional difference among these subclasses. Furthermore, the production of these three subclasses of IgZ might attribute to the differentiation and maturation of B lymphocytes residing within the systemic lymphoid tissues and mucosal associated lymphoid tissues. The subpopulations of IgZ positive B lymphocytes need further investigation.

Although much information on IgZ/T characterization has been achieved, few data are available on the *in situ* detection of IgZ-expressing cells in fish especially during the ontogeny[10, 23, 24]. Based on the analysis of Ig expression during the ontogeny by qRT-PCR, *in situ* hybridization (ISH) has been employed to show the CdgZ3-expressing cells and IgM-expressing cells in the tissues of carp at the development stage of 65 dpf. The ISH showed clearly the location of CdgZ3-expressing cells in the spleen, gill and hindgut which was similar to that of IgM-expressing cells, however, the amounts of transcripts of the two antibodies were different as their positive reactivity varied. These obtained results implied that we might reveal the distribution and location of CdgZ3-expressing cells by ISH even in the early development stage of common carp, which might help to clarify the production of CdgZ3 and the maturation of CdgZ3 positive B lymphocytes.

Conclusion

In conclusion, the third member of common carp CdgZ3 was cloned and characterized and the study about the expression and location of CdgZ3 compared with IgM, IgZ1 and IgZ2 in larvae ontogeny was performed. Comparative analysis between CdgZ3 and IgM in system immune tissues and gut associated lymphoid tissues of the common carp challenged by *A. hydrophila* injection and immersion was completed. We hope it might help to expand the information of adaptive immunity in common carp and teleost fish. Further research is required to understand the role of different IgZ/T subclasses and their corresponding positive B lymphocytes.

Methods

Ethics statement

For all experiments on live animals, we confirm that all methods were carried out in accordance with relevant guidelines and regulations. The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University (Permit Number: AEECSNU2017004). All efforts were made to minimize suffering. The study was carried out in compliance with the ARRIVE guidelines.

Experimental animals

Common carp (*Cyprinus carpio*) weighing between 150 g to 200 g were obtained from the Fresh Water Fishery Research Institute of Shandong Province (China) and maintained in tanks with aerated freshwater at 22–25°C. The fish were acclimated to the aquarium tanks for at least 2 weeks before being used in experiments. Fish were anesthetized by immersion in a solution of Tricaine (MS222, Sigma Aldrich) and killed. Tissue samples of blood, head kidney, spleen, liver, gill, foregut, midgut, hindgut, oral epithelium, skin, gonad, and muscle were collected immediately, frozen in liquid nitrogen, and stored at -80°C until use [25, 26]. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Shandong University.

Molecular cloning and analysis of common carp CclgZ3

Total RNA extraction and first-strand cDNA synthesis

Each frozen sample was ground in a mortar with nitrogen and then total RNA was isolated using the TRNzol Universal reagent (Tiangen, China). The quantity and quality of the total RNA were assessed using the NanoDrop Spectrophotometer (Thermo Scientific, USA) on all samples. The first-strand cDNAs were synthesized from 2 µg total RNA according to the manufacturer's instructions of FastQuant RT Kit (With gDNase) (Tiangen, China) [27, 28]. Total RNA from the collected samples was extracted following the procedure above and then the cDNA was stored at -80 °C until used in real-time quantitative PCR (RT-qPCR).

Molecular cloning and sequencing of common carp CclgZ3

The common carp *CclgZ3* cDNA fragment was firstly amplified by PCR with primer IgZ3 F1 and IgZ3 R1 designed based on the known sequences of teleost fish immunoglobulin Z (GenBank Accession No.: *Danio rerio* AY643750, EU732710.1, AY643750, *Ctenopharyngodon idella* DQ478943, GQ201421, *Cyprinus carpio* AB004105, AB598367, AB598368, AB598369). Head kidney cDNA of common carp was used as the template. Reaction steps were: 3 min initial denaturation at 94 °C, 1 min denaturation at 94 °C, 30 s annealing at 55 °C, 1 min extension at 68 °C (35 cycles), and 5 min final extension at 68 °C. Ex Taq HS (TaKaRa) was used in PCR and PCR products were loaded on 1% agarose gel and visualized by staining the gel in 0.1 mg/ml ethidium bromide. The DNA amplified in each reaction by PCR was purified using a Gel Extraction Kit (Tiangen), inserted into the pMD19-T vector (Takara) and transformed into competent TOP 10 *E. coli* cells for sequencing.

Subsequently, rapid amplifications of cDNA ends (RACE) was performed using the 3'-Full RACE Core Set (Takara) and the SMARTer RACE cDNA amplification Kit (TaKaRa) to get the full-length *CclgZ3* cDNA

sequences by the specific primers which were designed based on the obtained partial sequence. The 3'-Full RACE Core Set (TaKaRa) was utilized for obtaining 3'-unknown regions with the specific forward primers and the adaptor primers listed in Table 1. The first round of PCR was performed using the primer pair of IgZ3 race-3'outer primer/3'RACE outer primer, under the following conditions: one cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, followed by a final extension of 72°C for 10 min. The resultant product was diluted and re-amplified in the second round PCR using the primer pair of IgZ3 race-3'inner primer/3'RACE inner primer under the same reaction conditions. All PCR products were purified using a Gel Extraction Kit (Tiangen) and then were cloned into the pMD19-T vector (TaKaRa) for sequencing. For the 5' RACE reaction, the first strand cDNA was synthesized from 1 µg of total spleen lymphocyte RNA after adding the 5' RACE adapter to RNA, following the manufacturer's instruction. From the 5' RACE Ready cDNA samples, PCR reactions were performed using the Advantage 2 PCR Kit (Clontech), using the manufacturer's specifications and the specific primers shown in Supplemental Table 1. Reaction steps were: one cycle of 95°C for 1 min, 30 cycles of 95°C for 30 s and 68°C for 3 min, followed by a final extension of 68°C for 3 min. All PCR products were purified using a NucleoSpin Gel and PCR Clean-Up Kit (Takara), and then were cloned into the pRACE vector (TaKaRa) for sequencing.

Bioinformatic analysis of common carp CclgZ3

The full length of *CclgZ3* was confirmed by RT-PCR using sequence-specific primers IgZ3-1817F2/R2, IgZ3-1817F3/R3. The open reading frames (ORFs) and deduced protein sequences of *CclgZ3* was predicted using an ORF Finder program, and also by blasting genomic stretches against protein databases at NCBI (blastx). [29]. The location of Ig domains was predicted using the InterProScan program, Prosite Database and NCBI Conserved Domains. Post-translational modifications were predicted through the NetNGlyc 1.0 program. The theoretical isoelectric point and the molecular weight of the amino acid sequence were calculated using the Expasy Compute pI/Mw program. Multiple sequence alignment was conducted using CLUSTAL X version 2.1 with default parameters and was adjusted manually[11]. Based on the alignment, the phylogenetic tree was generated based on the deduced amino acid sequence using the Neighbour-Joining method with MEGA-X. All the sequences used for the phylogenetic analysis were listed in Table 2.

Gene expression studies of common carp immunoglobulins

Basal expression of immunoglobulin isotypes in organs of common carp

For the tissue expression, total RNA of the head kidney, spleen, liver, blood, skin, gill, foregut, midgut, hindgut, oral epithelium, gonad and brain from normal common carp were isolated and then reverse transcribed into first strand cDNA as described above. PCR was conducted with specific primers as indicated in Table 1 with SuperReal PreMix Plus (SYBR Green, Tiangen, China). The qPCR amplification program was 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 30 cycles to amplify S11 (as a standard) using primers S11F/S11R, and 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 30 cycles to amplify

S11 (as a standard) using primers for IgM, IgZ1, IgZ2, and CclgZ3 (listed in Table 1) in parallel tubes, respectively. The relative expression of the immunoglobulin gene was calculated and normalized against S11.

Basal expression of immunoglobulin isotypes in isolated leukocytes from different tissues of adult common carp

Whole Blood was collected from the caudal vein for isolation of PBL (peripheral blood lymphocytes) with a heparinized syringe and centrifuged at 4 °C, 500 x g for 10 min and then removed the serum. Diluted the cells 6-fold original volume of blood with RPMI-1640 medium at room temperature, then put on ice. Spleen was dissected from the anesthetized fish and placed in a sterile plastic culture dish containing 5 mL RPMI-1640 with 100U/mL penicillin G and 100 mg/mL streptomycin (Sigma, USA) respectively. Single-cell suspensions from the spleen were obtained by teased apart with sterile dissecting scissors at first, repeatedly aspirated and then passed through a 100 µm nylon mesh with RPMI-1640 medium. The total volume 10 mL of single-cell suspension was layered upon the same volume of Histopaque 1077 (Sigma, USA) in 50 mL centrifuge tubes gradually, centrifuged 500 x g for 40 min at 4 °C. Leukocytes were collected from the interface layer and washed three times with medium[30, 31]. Cell quantity and viability were determined by 0.4% trypan blue (Sigma, USA), and cells were collected for qPCR analysis[32]. The expression of immunoglobulin genes was calculated by the expression and normalized by S11.

Relative expression of immunoglobulin isotypes during the development of common carp

For the expression analysis of immunoglobulins during different development stages, fertilized eggs (n = 100) were obtained and the total RNA was extracted using the FastQuant RT Kit (With gDNase) (Tiangen) from embryos or larvae 1 day, 6 days, 10 days, 16 days and 31 days post fertilization, and then followed by the steps as described above. The Ig expression in tissues from common carp of 65 days post fertilization including gill, spleen, hindgut and liver were detected by qPCR following the same procedure.

In situ hybridization

Synthesis of RNA probes

Common carp IgM and *CclgZ3* cDNAs were amplified with primers (Table 1) and subcloned into the pSPT18 vector (Roche). The primers IgZ3_situ_F4 and IgZ3_situ_R4 were designed to amplify a 244 bp product corresponding to the constant region of the *CclgZ3* CH4 domain. The primers IgM_situ_F2 and IgM_situ_R2 were designed to amplify a 435 bp product corresponding to the constant region of common carp IgM CH4 domain. The cycling protocol was: denaturation at 94°C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58°C for 30 s and 72 °C for 30 s, with the final extension step of 72 °C for 10 min. The PCR products were visualized on 1% agarose gels containing ethidium bromide. The fragments were purified using TIANGel Midi Purification Kit (TIANGEN) and inserted into the pSPT18 vector (Roche) and

transfected into competent DH5α *E. coli* cells. Plasmid DNA from four clones was purified and sequenced respectively. The sequence analysis was performed to confirm the sequence identity and inserted orientation.

For generating RNA probes, the clones were digested with EcoR I or Hind III, and the fragments were purified on an agarose gel and used for *in vitro* transcription reactions with the DIG RNA Labeling Kit (Roche). Transcription was performed with SP6 RNA polymerase and T7 RNA polymerase according to the protocol so that antisense and sense RNA probes could be generated.

in situ hybridization

Spleen, gill and hindgut were aseptically extracted from the fish and fixed in 4% paraformaldehyde in PBS-H₂O_{DEPC} for at least 4 h. the tissues were then immersed in a 15% sucrose solution for 8 h and transferred in a 30% sucrose solution overnight. Embedded tissue in OCT was sectioned in 4 μm thickness and mounted onto poly-L-lysine coated slides. The slides were removed from the freezer, fixed in paraformaldehyde (4% in PBS, pH 7.4) for 20 min, washed three times with DEPC-treated PBS buffer (pH 7.4) and permeabilized with protein K (5 μg/ml) buffer at 37°C with gentle rocking. After washing in PBS-glycine buffer, the sections were washed twice with PBS-H₂O_{DEPC}. Prehybridization was done by incubating the sections with prehybridization buffer (Servicebio) for 60 minutes at 37°C. DIG-labeled antisense RNA probes (1 μg/ml) were applied with hybridization solution on the tissues and incubated at 55°C overnight in a moistened chamber. To remove the hybridization solution, the sections were washed sequentially by 5x SSC, 1xSSC, 0.5 x SSC and 20% formamide (50 min). the tissue sections were blocked by 5% serum blocking reagent for 30 min at room temperature. Anti-DIG-AP antibody used for the detection was diluted 1:500 in blocking buffer solution containing 5% serum. The sections were washed twice with PBS, subsequently, BCIP/NBT reagent (Roche) were applied according to the protocol. The reaction was visualized and documented using a bright-field microscope.

Organ-specific IgM and CclgZ3 expression after *A. hydrophila* challenge by different routes

Fifty common carp were divided into two groups for immune stimulation and challenged by *Aeromonas hydrophila* intraperitoneal (i.p.) injection and immersion respectively as previously described[27]. Briefly, The *Aeromonas hydrophila* used in the study was obtained from the China Center for Type Culture Collection and incubated in LB medium at 28°C overnight under continuous shaking. In the injection challenge, the *A. hydrophila* was inactivated in 0.5% formalin at 4 °C overnight and then was suspended in sterile 0.1M phosphate-buffered saline (PBS). Each fish was challenged by i.p. injection of 5 × 10⁷ CFU per fish. In the immersion challenge, the cultured *A. hydrophila* was added to the aquarium tank to the concentration of 1 × 10⁸ CFU/ml. After being treated for 40 min, the carp were taken out and transferred to a tank containing fresh water. On day 0, 1, 7, 14, 21 and 28 after stimulation, three fish from each group were anesthetized with Tricaine (MS-222) and the tissues samples including spleen and hindgut were taken and frozen in liquid nitrogen for total RNA extraction and then were detected by qPCR following the same procedure. Relative expression of immunoglobulin mRNA was determined using

relative quantification by the comparative method $2^{(-\Delta\Delta Ct)}$, the level of target mRNA was normalized with respect to S11, an internal reference gene, and the results were expressed relative to the unchallenged fish as control (denoted by 0 day)[32].

Abbreviations

Ig: immunoglobulin

MALT: mucosa-associated lymphoid tissues

GALT: gut-associated lymphoid tissue

Declarations

Ethics approval and consent to participate

For all experiments on live animals, we confirm that all methods were carried out in accordance with relevant guidelines and regulations. The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University (Permit Number: AEECSNU2017004). All efforts were made to minimize suffering. The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article is available in the GenBank (<https://www.ncbi.nlm.nih.gov/nuccore/1806102469>) and the accession number is MN170744.1.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

GWY, LGA and FMZ participated in the design of the study, MJL, CL, GCW, CW and YMW performed the experiments including gene clone, RACE, Real-time quantitative PCR, in situ hybridization and collected data, FMZ wrote the manuscript text and prepared figures and tables. All authors reviewed and approved the final manuscript.

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Not applicable.

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Tables

Due to technical limitations, table 1,2 is only available as a download in the Supplemental Files section.

Figures

OCTTCACTOCTATAGGGCAGCAGTGGTATCAAAGCAGAGTACATGGGGAGTCAGTGGGTGTGTCCAGTGATTAAAAACAACACAGTGTAACATCACTGTGTT 102

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JCH1

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JCH2

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K V E Y E K P S T T I S C A V I H A N M D N R R A P L Q V S T S Q 540

Jvec tail

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S K Q K E V S C D * 549

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CTCAAGACTAAATGTTTAAATTTGATAATTTACTTACATGTTGCATGTAAGTAAATATGGTCACATGAAAACATGTGCAGAATGATATCATAAAGCTTCTTTAC 2041

GTCTGTGTTGTGCTTACTGTACTGCTGTTAGTCTATAATAAATATAACATGCCATTTTTGTGTTGTTTACAAGCAATAAATAATTTGTTCCGATGAAAAAATAA 2144

Figure 1

Nucleotide and deduced amino acid sequence from secreted form of common carp (*Cyprinus carpio*) immunoglobulinZ3 (CclgZ3, GenBank Accession No. MN170744.1). (A) In the nucleotide sequence, the start codon (atg) and the stop codon (taa) are indicated in red. The typical polyadenylation signal (AATAAA) located upstream of the poly(A) tail is indicated in red. (B) In the amino acid sequence, the signal peptide (SP) is marked in green, the framework (FR) is shown in blue and the complementarity

determining regions (CDR) are shown in red. (C) The immunoglobulin domains CH1, CH2, CH3, CH4 and secreted tail are indicated by slash above the nucleotide sequence. (D) Potential N-glycosylation sites are underlined.

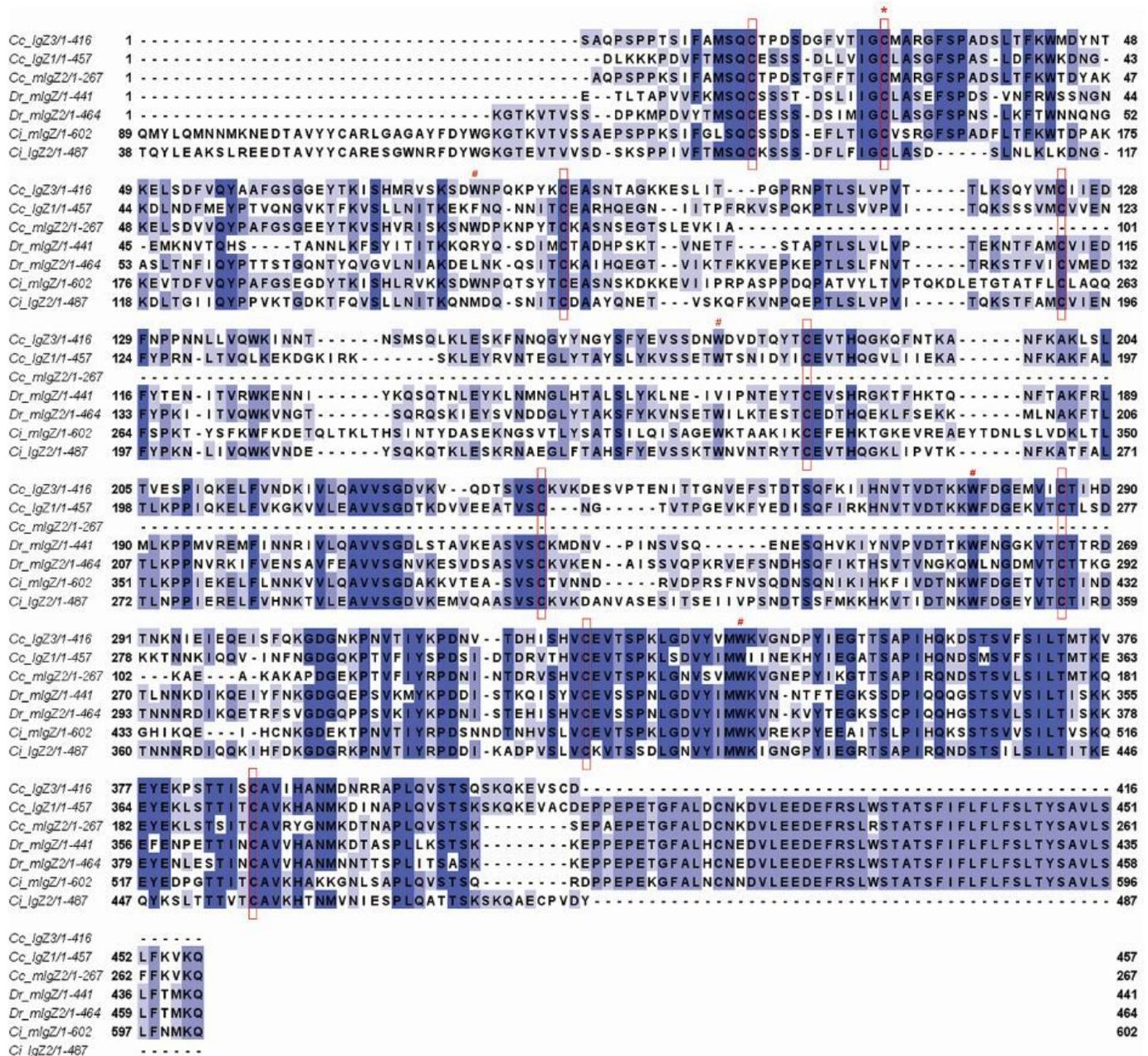


Figure 2

Alignment of the deduced amino acid sequence of CcIgZ3 homology from different species. Multiple sequence alignment was performed using CLUSTAL X version 2.1 with default parameters. The conserved cysteine (C) residues that formed the intrachain disulfide bonds are marked with box, the first conserved cysteine (C) within CH1 domain for interchain disulfide bond formation is indicated as an asterisk. The conserved tryptophan residues for the folding of IgSF domain are marked by hash. The GenBank Accession Number of the IgH sequences used for alignment are: Ci_IgZ (ABY76180), Ci_IgZ2 (ABF19723), Dr_IgZ2 (ACH92959), Dr_IgZ (AAT67444), Cc_IgZ1 (BAJ41037), Cc_IgZ2 (BAJ41038).

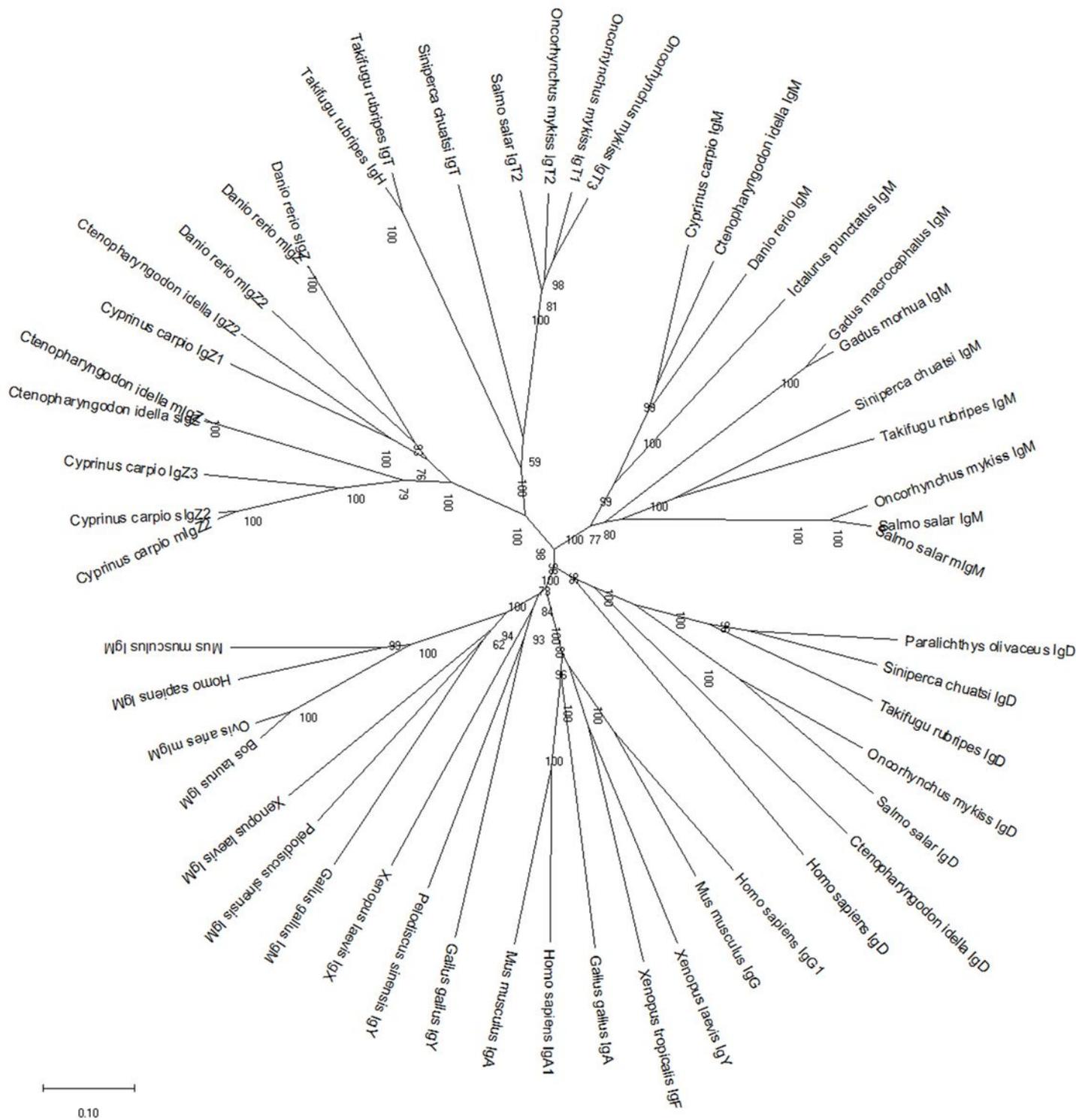


Figure 3

Phylogenetic analysis of vertebrate IgH based on CH sequences. The phylogenetic tree was obtained from a CLUSTALW alignment and MEGA X by Neighbor-Joining method with pairwise gap deletions. The scale indicated the genetic distance. Teleost Ig Clusters of of sequences are highlighted with a different color. The GenBank accession number are listed in Table 2.

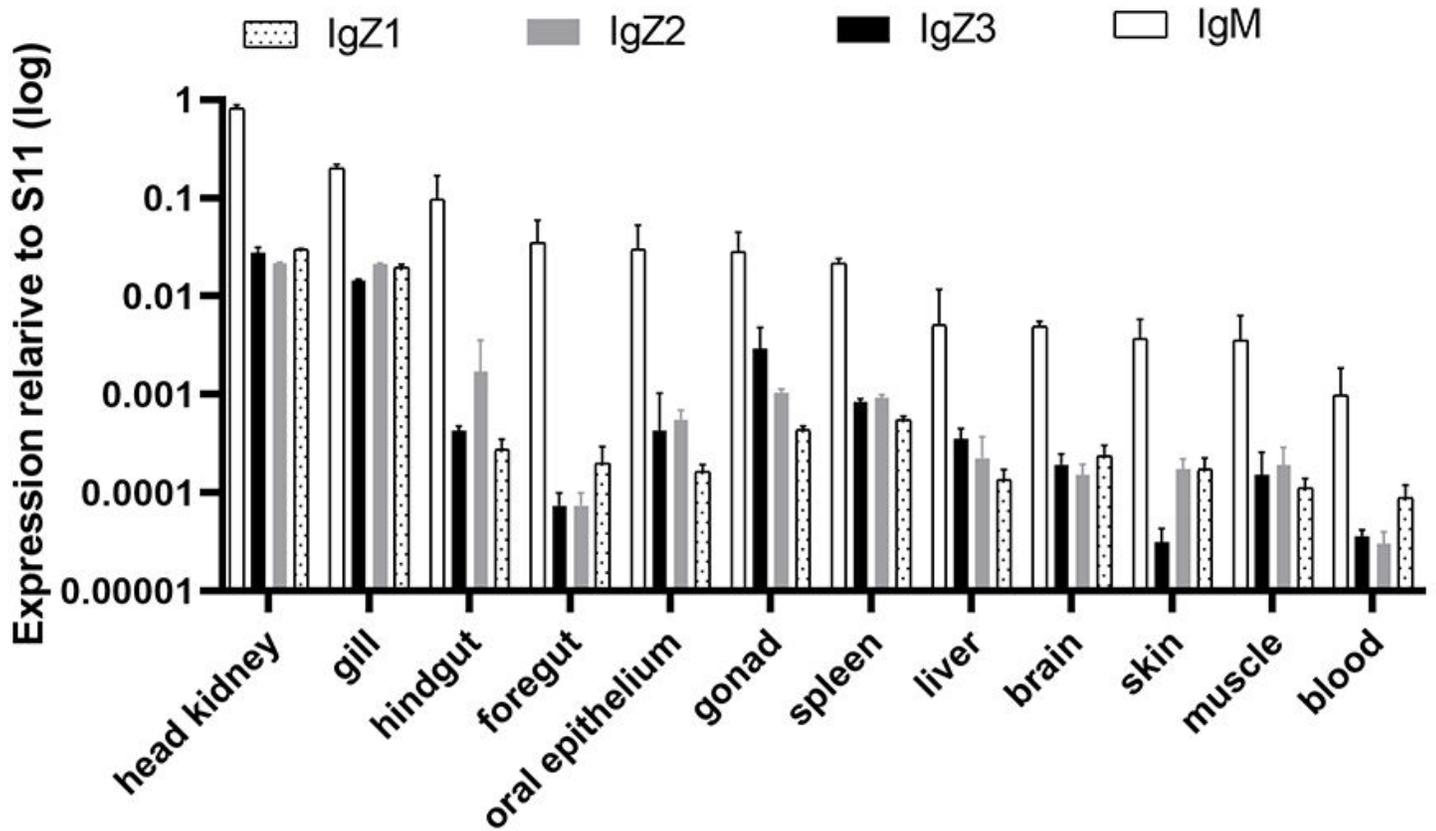


Figure 4

Basal gene expression of immunoglobulins in adult common carp. Expression of IgZ1, IgZ2, CclgZ3 and IgM in head kidney, gill, hindgut, foregut, oral epithelium, gonad, spleen, liver brain, skin, muscle and peripheral blood cells. Gene expression was measured by qRT-PCR normalized to the gene expression of S11. Averages and standard deviation of n=3 are plotted.

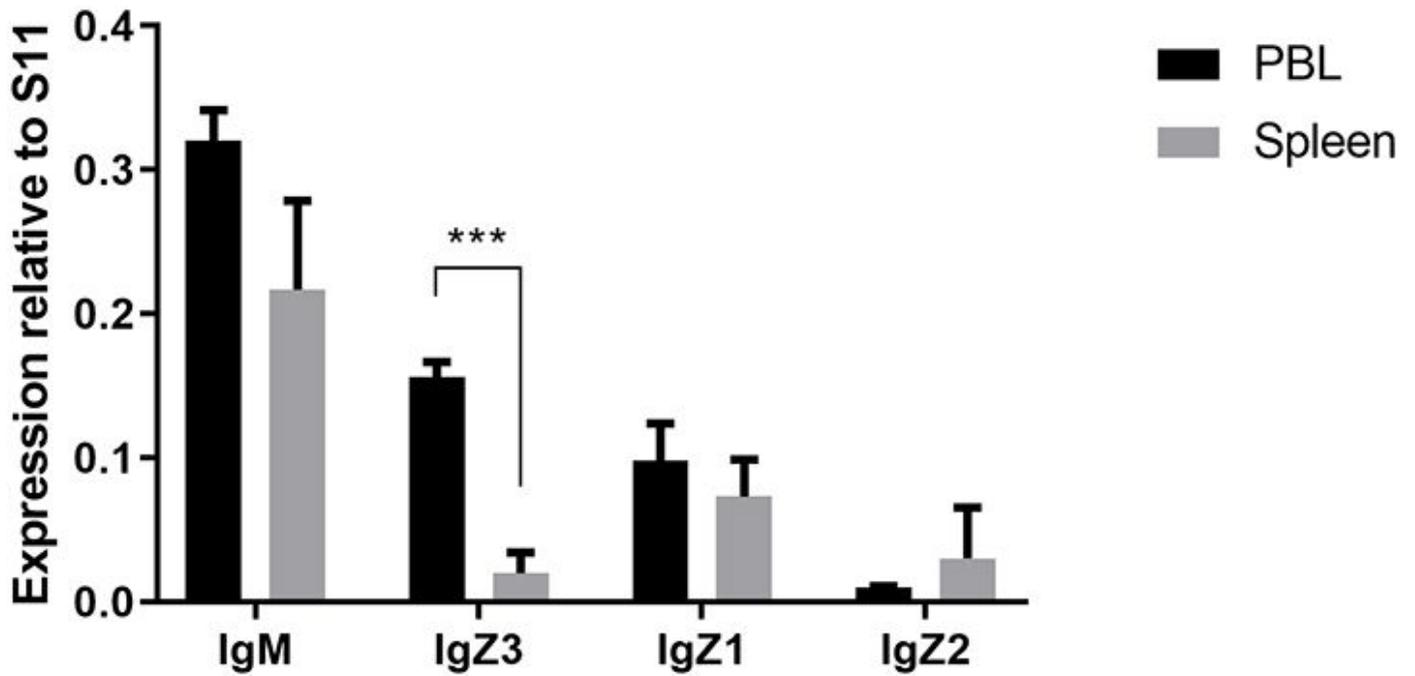


Figure 5

Constitutive immunoglobulin transcription levels in leukocytes of common carp. IgZ1, IgZ2, CclgZ3 and IgM mRNA levels were measured by qRT-PCR in Histopaque 1077 isolated lymphocytes from different tissues (spleen and peripheral blood cells) obtained from naive adult common carp. Results are shown as the mean gene expression relative to the expression of internal control S11 + SD. Averages and standard deviation of n=3 are plotted.

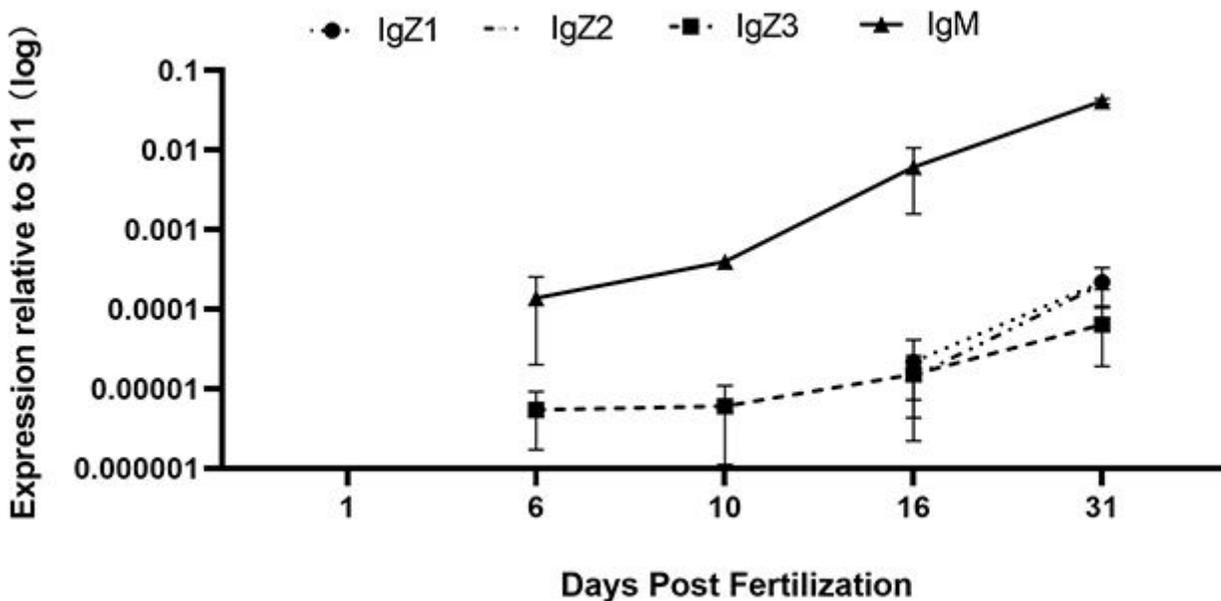


Figure 6

Ontogeny of immunoglobulins in common carp by gene expression studies in whole fish. Relative expression of immunoglobulins from 1 to 31 days post fertilization. Gene expression was measured by qRT-PCR and amplification of S11 is performed as an internal control. Averages and standard deviation of n=5 are plotted. RNA was pooled from 1 to 10 whole fish.

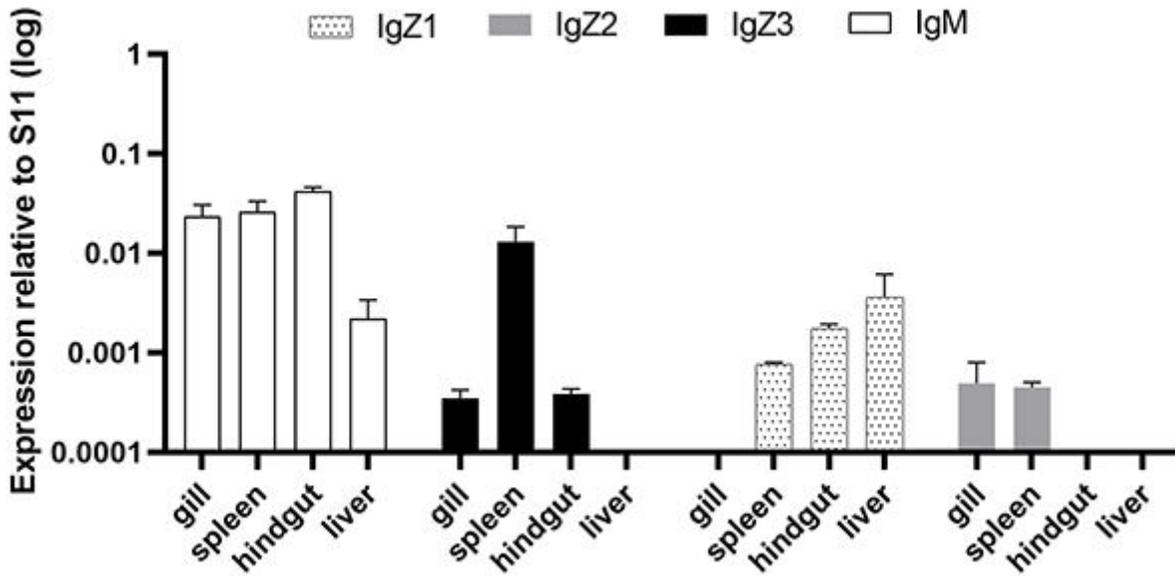


Figure 7

Expression of immunoglobulins in specific organs of common carp at the development stage of 65 dpf. Antibody expression in gill, spleen, hindgut and liver at 65 days post fertilization. Gene expression was measured by qRT-PCR normalized to the gene expression of S11. Averages and standard deviation of n=5 are plotted.

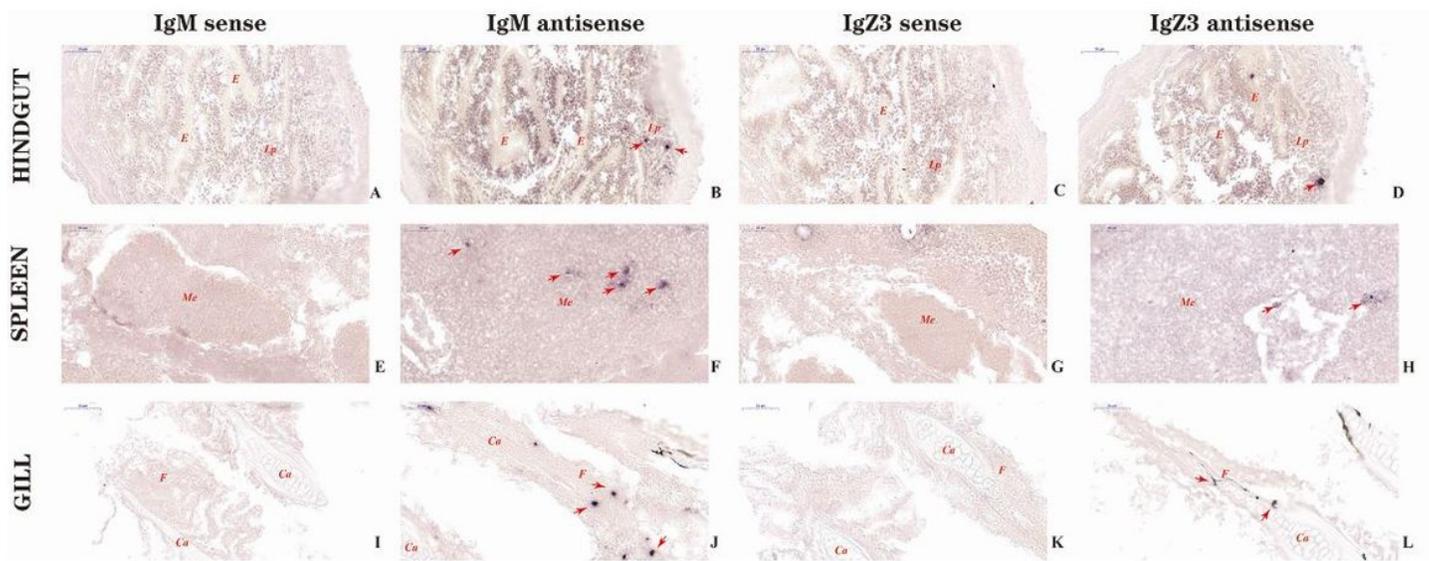


Figure 8

CclgZ3 and IgM transcripts are differentially expressed in spleen, gill and hindgut from developing common carp (65dpf). Sections of spleen, gill and hindgut from developing fish (65dpf) were hybridized with IgM sense (A, E, I) or antisense RNA probes (B, F, J), CclgZ3 sense (C, G, K) or antisense RNA probes (D, H, L). Lp: laminae propria, E: epithelium, Me: Medulla, Ca: cartilaginous cells, F: gill filament, Scale bars represent 50 μ m. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

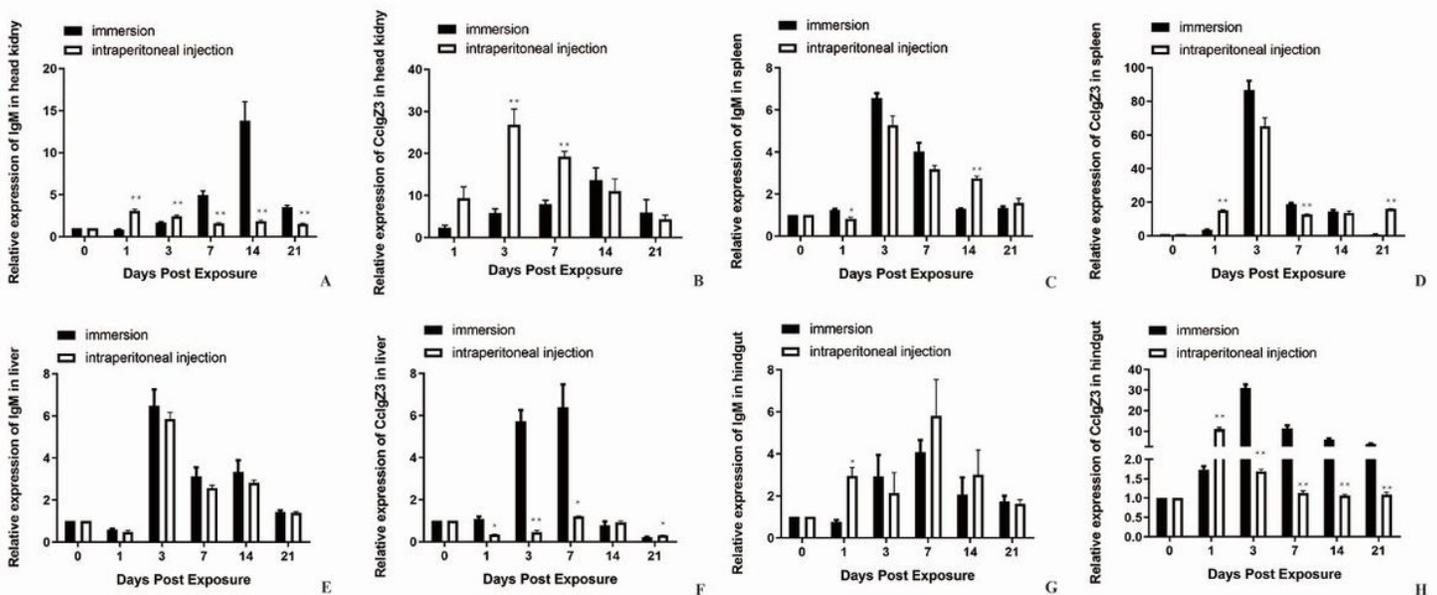


Figure 9

Expression analyses of immunoglobulin CclgZ3 and IgM genes in different organs of common carp challenged by *Aeromonas hydrophila* intraperitoneal injection and immersion. Time-dependent expression pattern of IgM in head kidney (A), spleen (C), liver (E), hindgut (G) and CclgZ3 in head kidney (B), spleen (D), liver (F), hindgut (H) after the challenge by intraperitoneal injection and immersion. Relative expression levels were detected by qRT-PCR and normalized against the expression at 0 day. S11 was used as internal controls. The values represent the mean \pm SD of each individual fish (n=3). Asterisks indicate significant differences between the intraperitoneal injection group and immersion group at each time point after the challenge, the p values were calculated by Student's t-test (*, $p < 0.05$)

Supplementary Files

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- [Table1..pdf](#)
- [table2.pdf](#)