

Aeromonas Hydrophila Infection Activates Death Receptor Apoptosis Pathway in the RBCs of Grass Carp (*Ctenopharyngodon idellus*)

Zhijie Lu

Zhongkai University of Agriculture and Engineering

Minxuan Yang

Zhongkai University of Agriculture and Engineering

Kai Zhang

Zhongkai University of Agriculture and Engineering

Fanbin Zhan

Zhongkai University of Agriculture and Engineering

Fenglin Li

Zhongkai University of Agriculture and Engineering

Fei Shi

Zhongkai University of Agriculture and Engineering

Yanan Li

Zhongkai University of Agriculture and Engineering

Lijuan Zhao

Zhongkai University of Agriculture and Engineering

Jun Li

Lake Superior State University Official Bookstore

Li Lin

Zhongkai University of Agriculture and Engineering

Zhendong Qin (✉ qinzhendongsc@163.com)

Zhongkai University of Agriculture and Engineering

Research

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Abstract

Background: Fish blood contains RBCs (Red blood cells) as the major cells. Unlike the absence of nucleus in mammalian RBC, fish RBC contains a nucleus. Previous researches have indicated that fish RBCs have a significant function in immune responses. However, the mechanism underlying the immune responses against bacterial infection of teleost RBCs remains enigmatic. To decipher the mechanisms, after the infection of *A. hydrophila*, transcriptomic profiling of grass carp RBCs was analyzed.

Results: Outcomes demonstrated that there were 2144 altogether DEGs (Differently Expressed Genes) between the *A. hydrophila* non-disease groups, which includes 817 up-regulated and 1327 down-regulated DEGs. Differently Expressed Genes were allocated to 45 GO terms, including 20 natural procedure terms, 14 cell related element, and 11 terms related to molecular functions. Likewise, the expression levels of cytokines by Quantitative Reverse Transcription Polymerase Chain Reaction showed that they (e.g. CCL4, CCL11, CCL20, IL-4, IL-12, and IFNa) were significantly increased after *A. hydrophila* infection, which was identical to the expression patterns of transcriptomic data. The infection could cause the apoptosis of RBCs which was confirmed by annexin V/PI assay. To further elucidate the apoptosis pathway, the expression levels of genes by mRNA included in cellular apoptosis were monitored. The outcomes indicated that the expressions of mRNA of p53, Fas, Leucine-rich repeats, death domain-containing (LRDD) and caspase 8 were all significantly increased, respectively.

Conclusions: Our results support the notion that *A. hydrophila* infection could activate Fas-related death receptor apoptosis pathway in grass carp RBCs, which will reveal another insight on the mechanisms underlying the antibacterial immunity of teleost fish RBCs.

Background

Ctenopharyngodon idellus is a significant cultured clean water fish around the world. The worldwide production of grass carp achieved approximately 5.5 Million tons in 2018 which is only after tilapia and becoming the second most cultured fish in the world [1]. The fast growth of high-density grass carp farming along with aquatic environmental deterioration has led to outbreaks of serious bacterial diseases, such as *A. hydrophila* infection disease [2]. The typical symptoms of *A. hydrophila* infection in grass carp are normally sepsis with systemic hemorrhage. The infection of *A. hydrophila* can cause high mortality, leading to devastating economic loss in the grass carp aquaculture industry [3].

Unlike RBCs of mammals, red blood cells of matured fish have a nucleus with organelles present in the cytoplasm [4]. Even though the principle function of red blood cell is the gas exchange, numerous other biological functions for red blood cells which nuclei are associated with immune responses have been recently uncovered [5, 6]. Nucleated RBCs can thus modulate leukocyte activity, respond to different pattern recognition receptors (PRRs), including peptidoglycan recognition protein (PGRP) receptor families [7], Toll-like receptors (TLRs) [8], and then release cytokines [9–11]. Therefore, fish RBCs are excellent models for exploring the immunity features of nucleated RBCs.

To promote functional genomics researches, NGS (Next-Generation Sequencing) also called high-throughput sequencing technology is frequently utilized, which includes new gene discovery, global gene expression, and SNP (single-nucleotide polymorphism) [12, 13]. By the quick advancement of high-throughput sequencing technologies, it provides valuable information for understanding host-pathogen interactions, including gene expression and immune responses [14]. By using high-throughput sequencing technologies, it has been reported that numerous of conserved and novel genes involved in innate and adaptive immunities during viral and bacterial infections in many fishes such as *Oncorhynchus mykiss* [15], *Oncorhynchus nerka* [16], *Schizothorax Prenanti* [17], *Oreochromis niloticus* [18], *Carassius Auratus* [19], *Hybrid Sturgeon* [20], *Common Carp* [21], and *Ctenopharyngodon idellus* [22]. Research on transcriptomic and proteomic of *O. mykiss* has indicated that RBCs possessing nucleus support many functions of immunity, e.g. cytokine expression, leukocyte activation, and antigen presentation [6]. Furthermore, NGS has been applied to distinguish the expression profile of the WBCs and RBCs of *Oreochromis niloticus* (Nile tilapia), and the results showed that suggested in response to poly(I:C) stimulation, RBCs expressed several immune genes [23].

Together, this information affirms the idea that fish RBCs have a considerable role in innate immunity. In our previous researches, we have reported that RBCs of grass carp showed antibacterial activity which is related to the generation of ROS and phagocytosis [24]. However, the underlying mechanism remains enigmatic. To decipher the mechanism, transcriptomic profiling of grass carp RBCs after *A. hydrophila* infection was analyzed in this research. The DEGs (differentially expressed genes) between the grass carp RBCs infected with or without *A. hydrophila* were determined. The differentially expressed genes were linked with numerous functional categories and immune signaling pathways which were important to antibacterial immunity, such as CCR interaction (cytokine-cytokine receptor interaction), Toll-like receptor signaling pathways (TLR signaling pathways) and TNF signaling pathways. Furthermore, transcriptomic profiling and qRT-PCR revealed that *A. hydrophila* infection could up-regulate the expressions of some cytokine genes and activate the Fas-related death receptor apoptosis pathway.

Materials And Methods

Experimental fish

A place supplied grass carp which weighs around 100-250 grams were situated in city of Guangzhou, province of Guangdong, China. These were kept at the temperature of 25-26 °C in a continuous flow water network for around 15 days so as they could acclimate to the laboratory conditions before the experiments were carried out.

Isolation and purification of RBCs

RBCs of grass carp were obtained from the peripheral blood as explained already with minor changes [25]. Briefly, heparinized syringes were used to draw peripheral blood from the caudal vein which is combined with 0.7% buffer saline solution (0.7 g NaCl in 100 ml H₂O) containing heparin sodium (0.1

mg/ml). The purification of red blood cells was done by two consecutive density-gradient centrifugations in 34% and 51% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) at 500 g for 35 minutes at 4°C. The RBCs were gathered and washed three times in a 0.7% buffer saline solution, and then resuspended in an L-15 medium (Invitrogen, USA) supplemented with 100 µg/ml streptomycin (Sigma, USA) and, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and at a density of 10^6 cells/ml at 28 °C. All grass carp tests were done in careful observance with the guidance of the Ministry of Science and Technology of China 2006 and affirmed by the Zhongkai University of Agriculture and Engineering's Animal Ethics Committee.

Infection of RBCs with *A. hydrophila*

A. hydrophila was maintained and cultured according to our routine laboratory protocols [24]. Subsequently, the experimental RBCs (1×10^6 cells/ml) from 6 fish were divided into control and the infected groups. The RBCs in the infected group were incubated at a 1:10 ratio with *A. hydrophila* for 12 hours under constant rotation at 28°C, whereas the RBCs in the control group were incubated with the similar amount of PBS. After 12 hr treatment, three equal specimens were combined as biological replicas.

Extraction, RNA-Seq library construction, and sequencing of RNA

Complete RNAs were removed from the *A. hydrophila*-infected and non-infected RBCs using RNAiso plus made in Takara, Japan as indicated by the maker's procedure. Subsequently, the characteristics and amount of the complete RNA were identified utilizing Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and Nano Drop 2000 (Thermo Scientific), respectively. The libraries were built using the TruSeq™ RNA Sample Prep Kit (Illumina, San Diego, CA, USA) according to the maker's directions. Afterward, the three RNA-Seq libraries were carried out with 150/125 bp paired-end sequencing on the BGISEQ-500 platform (BGI, Shenzhen, China) as per the last record [26].

Sequence filtering, gene annotation, and de novo assembly

The transcriptomic test was performed by the strategy described before head [27]. The generated raw sequencing readings were firstly clean up by eliminating adaptor sequences and reduced quality sequences (over 20% of the basic qualities were below than 10) using the tool FASTX, which is utilized to decrease data clamor and clean raw readings. The informational collection of the source of rRNA arrangements was acquired from general rRNA databases. The remaining clean readings were planned to a *C. idellus* reference genome utilizing HISAT. After mapping of genome, StringTie were utilized to rebuild transcripts, and with genome annotation information, the coding capability of those latest transcripts predicted utilizing CPC and the novel transcripts were identified using the Cuffcompare tool. Gene expression abundance was evaluated utilizing the RPKM (readings-per kilobase per million mapped reads) method. All reference genes were performed with a sequence based on the NCBI nr (non-redundant) protein with an E-value threshold of 10⁻⁵ and the Uniprot database. Subsequently, all

differential expressed genes (DEGs) were mapped to the GO terms data for functional annotation, the KOG, KEGG for pathway-enrichment analysis.

DEGs, KEGG, and GO enrichment analysis

Based on the obtained levels of gene expression, DEGs between *A. hydrophila* non-infected and infected groups were utilized with criteria with a FC (fold change) of gene expression > 2.0 (complete value of the log₂ Ratio ≥ 1) and FDR (False Discovery Rate) ≤ 0.001 . To know more about the DEGs and pathways responding to the infection of *A. hydrophila*, functional groups and pathways encompassing the DEGs were plotted to terms in the KEGG and GO databases with the Blast2 GO pipeline and KEGG enrichment analysis utilizing KOBAS software, respectively.

1. *hydrophila* infection and immune genes expression

Six cytokines genes (CCL4, CCL11, CCL20, IL4, IL12, and IFN α) were analyzed by qRT-PCR to further validate RNA-seq data. The reverse transcription reaction was in a total volume of 20 μ l, which contains 10 μ l AceQ® qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China), 1 μ l cDNA, 7 μ l DEPC treated water, and 1 μ l of each particular primer (list in Table 1). The quantitative real-time PCR cycling conditions were as stated: 95 °C for 3 minutes, further at 95 °C for 15 seconds of 40 cycles, for 30 seconds at 60 °C, for 20 seconds at 72 °C, and finally for 5 minutes at 4 °C [28]. All reactions of each gene in each sample were carried out in triplicate. Meanwhile, an analysis of melting curve was performed to identify the nature of target. The relative expression ratio of the β -actin gene versus the target genes was calculated using the $2^{-\Delta\Delta CT}$ method [29], and all the information was provided in terms of relative mRNA expression.

Examination of apoptosis in *A. hydrophila*-infected RBCs

The RBCs (1×10^6 cells/ml) were separated into two different groups; infected and control. The RBCs in the infected group were incubated with *A. hydrophila* at a ratio of 1:10 for 6, 12, and 24 h under constant rotation at 28 °C, respectively. The control group was incubated with the equal proportion of PBS. After 6, 12, and 24 h of infection, a kit called the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, USA) was utilized to test the apoptosis of RBCs as stated earlier [30]. After staining, the RBCs were viewed by fluorescence microscopy (Leica DMI8, Germany). Furthermore, to additionally explain the mechanism of apoptosis, some apoptosis pathway-related genes; including p53, Fas, LRDD, and caspase 8 were determined by qRT-PCR. All primers were shown in Table 1.

Statistical evaluation

Statistical evaluation was carried out by utilizing SPSS software version 17.0. Whole formation was depicted as a mean \pm SD (standard deviation) at least 3 separate tests. The statistical importance of the information was evaluated by ANOVA (one-way analysis of variance), and GraphPad Prism 7 was used to draw figures [31]. The difference was considered important when $P < 0.05$ (*).

Results

Transcriptomic sequencing and de novo assembly analysis

The cDNA libraries from the RBCs of grass carp with and without infection of *A. hydrophila* were ranked on the BGISEQ-500 platform. For each sample, 82.47% of the readings were effectively mapped to the genome of grass carp. The total de novo assembly readings from the library yielded total transcripts of 21,753 known genes and 1262 novel genes comparing to the control groups (Fig. 1B). The unigene size distribution is indicated in Fig. 1 (A), and the most plentiful unigenes were grouped in a cluster of \geq 3000 bp in length. Levels of gene expression were standardized to FPKM, a total of 20,765, and 18,612 contigs were detected from the control and *A. hydrophila*-challenged groups, respectively, with 18,501 common contigs in both (Fig. 1C). The unigene size distribution of the transcription factors (TF) family is indicated in Fig. 2, and the more common unigenes of TF were agglomerated in a ZF-C2H2 cluster.

Functional Explanation Of The Degs

To identify the profile of gene expression of the RBCs after *A. hydrophila* infection for 12 h, DEG libraries were built from the control and infected groups. The outcomes indicated that there were 2144 DEGs between the infected and control groups, including 817 up-regulated genes and 1327 down-regulated genes. As appeared in Fig. 3, these DEGs were characterized into three classes: cellular component, molecular function, and biological process. Between the up-regulated genes in the natural procedure, the most beneficial subclasses were “cellular process”, trailed by “biological regulation” and “metabolic process”. In the elements of cells, “the cell”, “organelle” and “membrane” were the majority of the 3 improved subclasses. Whereas, in the class of molecular functions, “binding” was the more beneficial subclass, followed by “catalytic activity” and “molecular function regulator” (Fig. 3A). Between the GO analyses of down-regulated genes in the biological procedure, the more beneficial subclass was the “cellular process”. The “cell” was the most enrichment subclass in the cellular component. However, “binding” had the most improved number in the class of molecular function (Fig. 3B).

Furthermore, to additionally comprehend the roles of the DEGs and the signaling pathways involved in *A. hydrophila* infection, DEGs in RBCs with *A. hydrophila* infection were visualized by a volcano plot. The blue-colored plots show important down-regulated genes, the red-colored plots show important up-regulated genes, and the gray plots represent no important changed genes (Fig. 4A). Correspondingly, the MA plot exhibited that the differential articulation designs were not because of the expression genes number (Fig. 4B).

Analysis Of Kegg Enrichment Pathways

The top 20 KEGG enrichment pathways were observed in *A. hydrophila* group. As shown in Fig. 5, there are 523 DEGs that are closely involved in antibacterial immune response, with the largest pathway of

Herpes simplex infection (57 DEGs), followed by CCR interaction (55 DEGs), Hepatitis B 38 DEGs, and Toll-like receptor signaling pathway (38 DEGs). Intriguingly, between these antibacterial pathways, three KEGG pathways directly associated with bacterial invasion performed further hierarchical clustering: CCR interaction (cytokine-cytokine receptor interaction), TNF signaling pathway, and TLR receptor signaling pathway (Toll-like signaling pathway) (Fig. 6).

Detection Of Immune-related Degs

To confirm the immune-related DEGs, six cytokines (CCL4, CCL11, CCL20, IL4, IL12, and IFN- α) were analyzed by qRT-PCR after the RBCs were challenged by *A. hydrophila* at multiple time points (6, 12 and 24 h). Compare with the control group, the expressions of all six cytokine genes were all considerably up-regulated at 24 hr after the *A. hydrophila* infection. However, the expressions of CCL20 and IL-4 were already significantly up-regulated both at 12 h after the infection (Fig. 7).

***A. hydrophila* infection induced apoptosis in RBCs**

Annexin V/PI apoptosis kit was used to test the apoptosis triggered by *A. hydrophila* infection. Indicated in Fig. 8, the green signal indicates early apoptosis; the red signal represents late apoptosis. The outcomes demonstrated that only few apoptotic cells were seen at 6, 12, and 24 h in the control groups. However, there were many apoptotic cells observed in the infected RBCs at 6, 12, and 24 h, with stronger red signal present at 12 and 24 hours, showing that prolonged infection may cause more severe programmed cell death (Fig. 8). To further address the apoptosis pathway, RNA-seq data were subjected to analyzed. The outcomes demonstrated that *A. hydrophila* infection could up-regulated the expressions of p53, Fas, LRDD, and caspase 8. The expressions of these 4 genes were further measured by qRT-PCR. As shown in Fig. 9, the mRNA expression of Fas was importantly up-regulated from 6 to 24 h post-infection, whereas the levels of mRNA of p53, LRDD, and caspase 8 were also increased from 12 to 24 h. Since these 4 genes were closely related with the death receptor apoptosis pathway, indicating that *A. hydrophila* infection can trigger Fas related death receptor apoptosis pathway. The potential mechanism was proposed in Fig. 10.

Discussion

For a long time, red blood cells have been portrayed as inactive onlookers as opposed to members in immune responses. Advances over the recent years have revealed that RBCs appear to play an unusual role in antimicrobial immunity. In our previous study, we have reported that RBCs of grass carp showed antibacterial activity which is related to reactive oxygen species and phagocytosis generation [24]. However, the underlying mechanisms remain enigmatic. To further decipher the mechanism of antimicrobial immunity, transcriptomic profilings of grass carp RBCs infected with or without *A. hydrophila* were compared and analyzed. A sum of 2144 DEGs among the control and *A. hydrophila* groups were recognized including 817 up-regulated and 1327 down-regulated genes. Furthermore, in

pathway-enrichment analysis, the main 20 KEGG enrichment pathways were obtained by *A. hydrophila* infection, 523 DEGs were closely involved in the antibacterial immune response.

In the transcriptomic analysis of human red blood cells, the different stages of development (reticulocytes, erythroblasts, and mature) characterization of mammalian RBCs showed a series of expressed genes linked with the immune defense [32]. Recently, some researches have explained the transcriptomic analysis of blood cells in teleost. An enormous number of genes engaged in immune response and apoptosis are differentially expressed in the *O. mykiss* of RBCs challenged at different temperatures [33]. After infected with piscine orthoreovirus in *Salmo salar* RBCs, the cells showed a major phenotypic change and were found to activate innate antiviral immunity [34]. Also, when the irresistible salmon anemia virus contaminated the RBCs of *Atlantic salmon*, RBCs would release cytokines to modulate leukocyte activity [11, 35]. A family of low molecular weight proteins-Cytokines is secreted by activated immune-related cells, which are induced by several pathogens such as bacteria, viruses and parasites [36]. Cytokines could be majorly grouped into ILs (interleukins), TNFs (tumor necrosis factors), chemokines, and IFNs (interferons) [37]. Cytokines play significant function in host defense and inflammation [38–40]. In this report, by the analysis of KEGG enrichment pathways, we observed that TNF signaling pathways and cytokine-cytokine receptor interaction were activated after the *A. hydrophila* infection in grass carp RBCs. The qRT-PCR results also showed that the mRNA levels of 6 cytokines (CCL4, CCL11, CCL20, IL4, IL12, and IFN- α) were all significantly up-regulated after RBCs were challenged by *A. hydrophila*. These results indicated that the RBCs can also perform antibacterial functions by releasing a series of cytokines, which share a similar function with leukocyte. However, the details of the regulation of these cytokines need to be further elucidated.

Programmed cell death or apoptosis has a broad range of physiological functions, including fighting against the infection [41]. Apoptosis mainly occurs as three distinct pathways: endoplasmic reticulum pathway, death receptor pathway, and mitochondrial pathway [42]. For death receptor pathway, external factors can activate a member of this family on the cell membrane (for example; Fas), resulting in a series of activations of proteins, including caspase 8 and caspase 3, and ultimately results in cellular apoptosis [43]. The mitochondrial pathway is triggered by mitochondrial membrane potential changes under the action of foreign factors, resulting in releasing C cytochrome, afterwards triggers caspase 3 and caspase 9, resulting in programmed cell death [44]. For the endoplasmic reticulum pathway, the imbalance of calcium ion concentration in the endoplasmic reticulum led to the triggering of caspase 12, which then triggers the caspase 3 and 9, and initiation of cell death [45]. Here, our result showed that the *A. hydrophila* infection could significantly induce cellular apoptosis in grass carp RBCs after determined by an annexin V/PI apoptosis kit. To further address the apoptosis pathway, RNA-seq data were subjected to analysis. The outcomes indicated that *A. hydrophila* infection could up-regulated four gene's expressions (P53, Fas, LRDD, and caspase 8) which were closely related with the death receptor apoptosis pathway, indicating that *A. hydrophila* infection could activate Fas-related death receptor apoptosis pathway. Interestingly, the Fas and caspase 9 genes of apoptosis-related pathways were the most over-expressed in RBCs of rock bream infected with a megalocytivirus [46].

Based on the previous study in mammals [47], it tempts us to speculate that *A. hydrophila* infection might activate p53, subsequently activates the Fas trimerizing, which in turn results in the specialized adaptor protein's recruitment and cause oligomerization of caspase to activate 8. Thereafter, the oligomerized caspase 8 directly splits and caspase to become active 3, which eventually leads to the cell death (Fig. 10). However, the detailed mechanism needs to be elucidated in the future. It does not escape our notice, we also observe that after *A. hydrophila* infection, the expression of caspase 9 was also up-regulated (data not show), showing that *A. hydrophila* infection may likewise enact and may contains other cellular apoptosis pathways in grass carp RBCs after bacterial infection, however, the exact mechanisms remain to be further researched.

Conclusions

To summarize, we have shown that the RBCs of grass carp can produce immunological reactions to *A. hydrophila* infection. These reactions were characterized by the up-regulation of cytokines and activation of the death receptor apoptosis pathway. These findings give new bits of knowledge into a further comprehension of the system of anti-*A. hydrophila* immunity in grass carp and add to mitigating an economically significant bacterial infection influencing aquaculture of grass carp.

Abbreviations

RBCs

Red blood cells; DEGs:differently expressed genes; LRDD:Leucine-rich repeats and death domain containing; PRRs:pattern recognition receptors; PGRP:peptidoglycan recognition protein; TLRs:Toll-like receptors; SNP:single-nucleotide polymorphism; NGS:next-generation sequencing; WBCs:white blood cells;

CCR

cytokine-cytokine receptor; qRT-PCR:quantitative real-time PCR; FBS:fetal bovine serum; RPKM:readings-per-kilobase-per-million-mapped-reads; nr:non-redundant; GO:Gene Ontology; FC:fold change; FDR:False Discovery Rate; SD:standard deviation; TF:transcription factors; ILs:interleukins; TNFs:tumor necrosis factors; IFNs:interferons; CCL:CC chemokines.

Declarations

Ethics approval and consent to participate

All grass carp tests were done in careful observance with the guidance of the Ministry of Science and Technology of China 2006 and affirmed by the Zhongkai University of Agriculture and Engineering's Animal Ethics Committee.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Li Lin, Zhendong Qin conceived and designed the project; Zhijie Lu, Minxuan Yang participated in data analysis and figure preparation; Zhijie Lu, Minxuan Yang prepared the manuscript; Kai Zhang, Fanbin Zhan, Fenglin Li, Fei Shi, revised the manuscript; Yanan Li, Lijuan Zhao, Jun Li participated in discussions and provided suggestions.

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Authors' information

¹Guangdong Provincial Water Environment and Aquatic Products Security Engineering Technology Research Center, Guangzhou Key Laboratory of Aquatic Animal Diseases and Waterfowl Breeding, College of Animal Sciences and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong Province, 510222. ²School of Biological Sciences, Lake Superior State University, Sault Ste. Marie, MI, 49783, USA

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Tables

Table 1

List of primers used for RT-qPCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
CCL4	TGCTGTTCTCCTATACGGATCG	TTTCTGGATGAGCTTCTGTACCC
CCL11	CTAGAGTTGGAGTGACATGCTGT	AGCAGTACTTGTCCCTCTGTG
CCL20	CTTCAACCTGCCTTGTCTCAAG	CGGAGCAAAAAGACAATCCTCTG
IL4	CTCTTGGCACTGACATTGTAGC	GGTTATGTAGGGTCTGGTCACA
IL12	GCACTCACGGTACTGTCCCTAAAT	CTAACGACTGTTTGGTGAGCTC
IFN	GATAAGCAACGAGTCTTGAGCC	GAATTCATGTGCTCTGGCATC
Fas	CATGTTCCCTTTCATCCCCAGAA	TGGAAGACAGTGAGGAAAAGGAG
Caspase8	GTTTCTGTGCATGGACCTGATT	CAGCTCTTTCCACTTCCTCTT
p53	CCAGACCTGAACAAGCTTTTG	GAGCCAATCCATCAGTATCAGGT
LRDD	GTTCTACCAGAGGATTGGCAGA	GAGCTGGTCATTTCGATAGC
β-actin	ACCCACACCGTGCCATCTA	CGGACAATTCTTTGGCTG

Figures

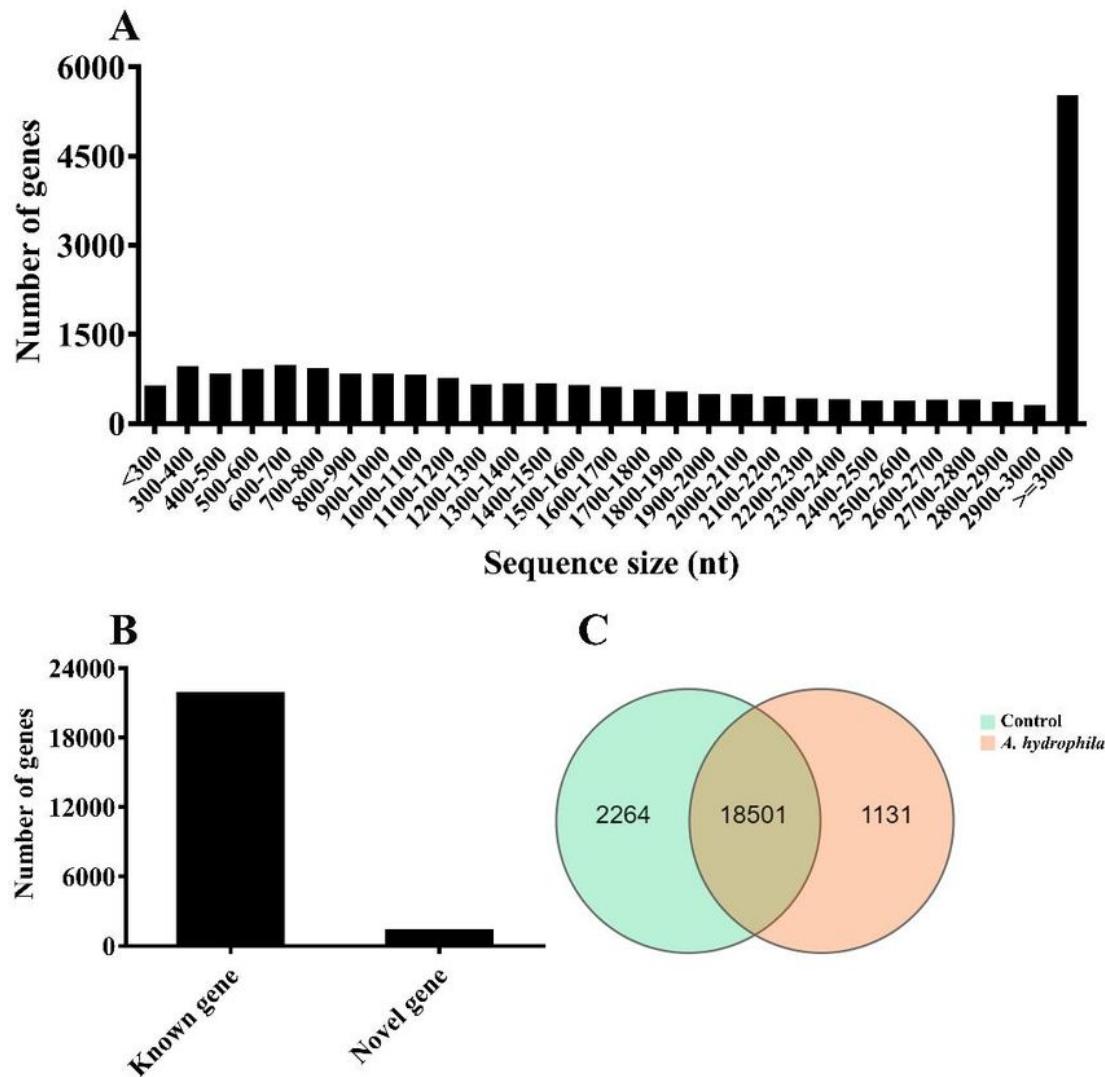


Figure 1

(A) Overview of *A. hydrophila* infected grass carp RBCs transcriptomic sequence length distributions for all unigenes. The x-axis indicates the length interval of transcript and the y-axis indicates the number of transcripts with different lengths. (B) The number of known genes and novel genes. (C) The Venn diagram of control and *A. hydrophila* intergroup expression.

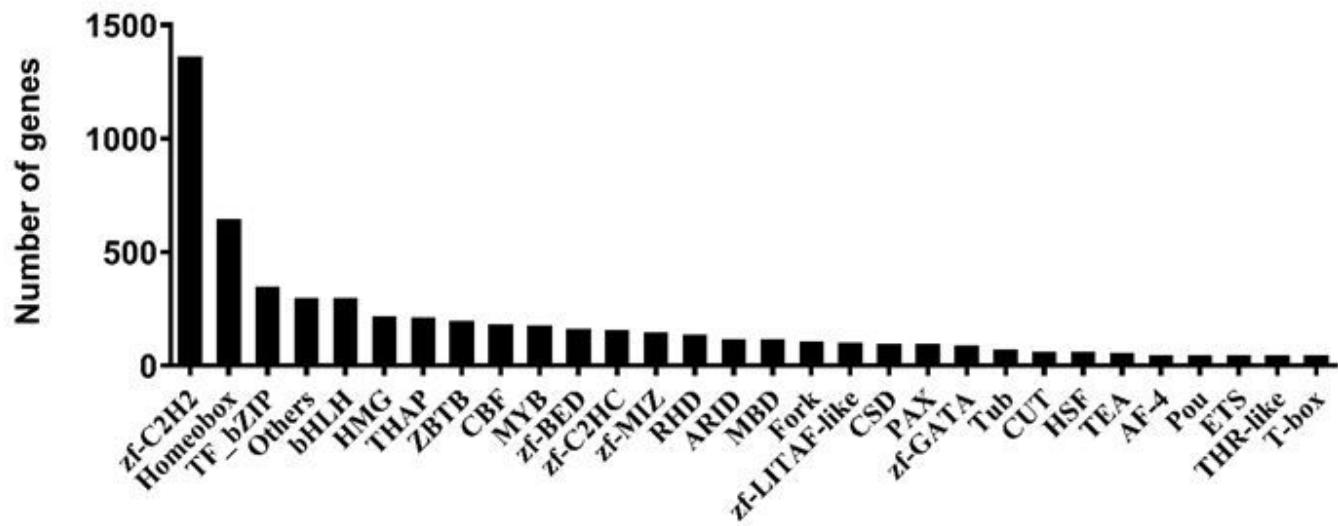


Figure 2

Classification of transcription factor families for all transcriptomic genes, such as AF-4, AP-2, ARID, C/EBP, CBF, CG-1, COE, CP2, CSD, CSL, CTF/NFI, CUT, DM, E2F, ESR-like, ETS, Fork, GCM, GCNF-like, GTF2I, HMG, HMGI/HMGY, HPD, HSF, HTH, Homeobox, IRF, MBD, MH1, MYB, Miscellaneous, NCU-G1, NDT80/PhoG, NF-YA, NF-YB, NF-YC, NGFIB-like, Nrf1, Others, P53, PAX, PC4, Pou, RFX, RHD, RXR-like, Runt, SAND, SF-like, SRF, STAT, T-box, TEA, TF_Otx, TF_bZIP, THAP, THR-like, TSC22, Tub, ZBTB, bHLH, zf-BED, zf-C2H2, zf-C2HC, zf-GAGA, zf-GATA, zf-LITAF-like, zf-MIZ, and zf-NF-X1.

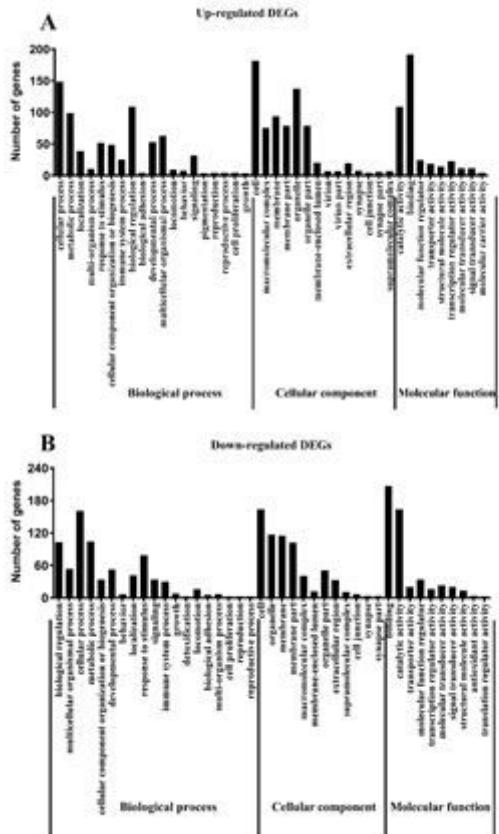
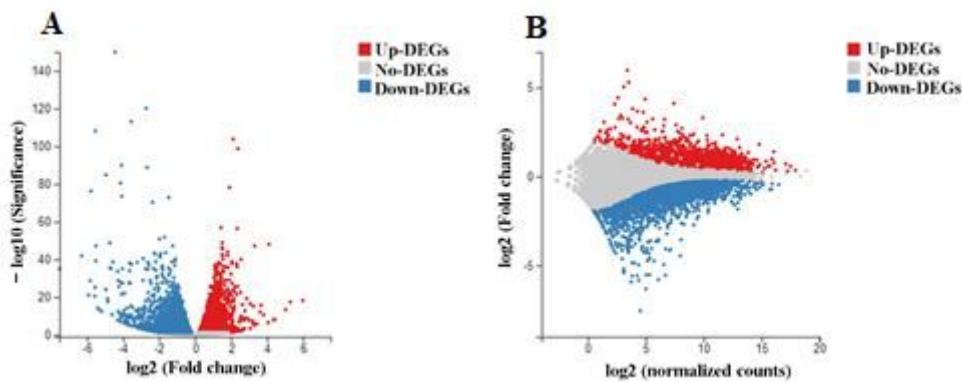
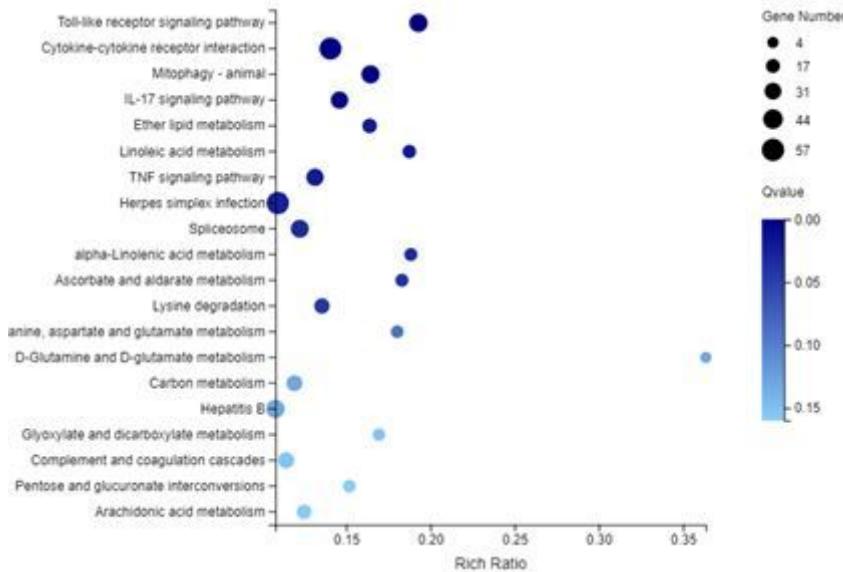


Figure 3

Gene ontology enrichment analysis of DEGs in *A. hydrophila* infected grass carp RBCs. The differentially expressed genes (DEGs) were classified into three subclasses, including molecular function, cellular component and biological process. (A) The up-regulated genes of GO analysis DEGs upon *A. hydrophila* infection. (B) The down-regulated genes of GO analysis DEGs upon *A. hydrophila* infection.

**Figure 4**

A volcano plot of DEGs. The red pots indicate significant up-regulated genes, while the blue pots indicate significant down-regulated genes and the gray pots represent no significantly changed genes. (A) The x-axis represents the difference multiple value after log2 conversion, the y-axis represents the significant value after -log10 conversion. (B) X axis represents the value of average expression level after log2 conversion, Y axis represents the value of multiple of difference after log2 conversion.

**Figure 5**

Differential gene KEGG Pathway enrichment bubble map. The x-axis is the enrichment ratio, the y-axis is the size of the KEGG Pathway. The bubble indicates the number of genes annotated to a KEGG Pathway; the color represents the enrichment Q value. The darker color indicates the smaller Q value.

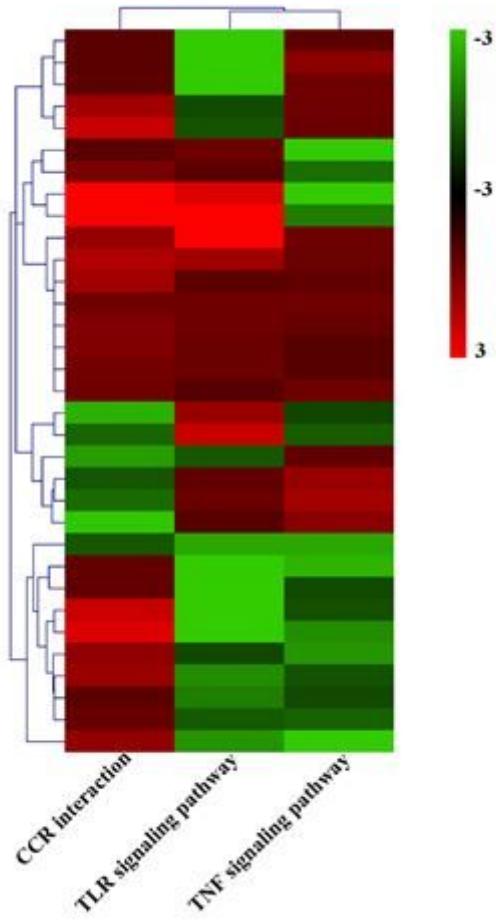


Figure 6

Heat map of partial immune-related DEGs in RBCs infected with *A. hydrophila* basing on the KEGG analysis. Intensity of color indicates expression levels.

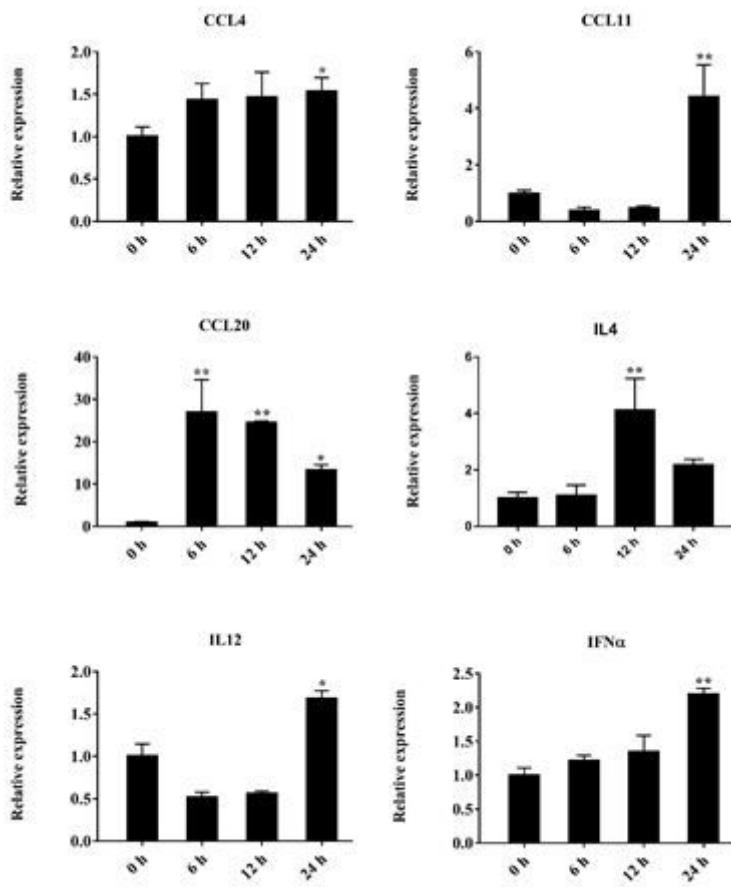


Figure 7

Validation of the transcriptomic DEGs using cytokine genes of grass carp RBCs infected with *A. hydrophila* at 6 h, 12 h and 24 h post of the infection. Gene expression was analyzed by RT-qPCR, $2^{-\Delta\Delta Ct}$ method, using endogenous gene β -actin as a reference. Data represent means \pm SD. Statistical comparison of the mRNA levels detected at different time points was carried out by one way-analysis of variance (* $P < 0.05$, ** $P < 0.01$).

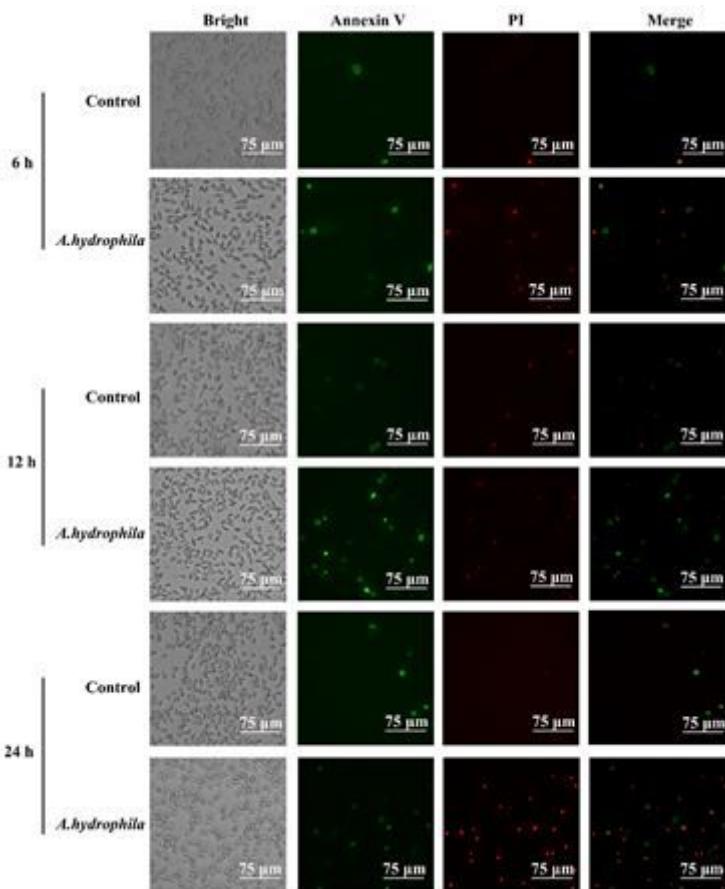


Figure 8

A. hydrophila infection induced apoptosis in grass carp RBCs at 6 h, 12 h and 24 h post of infection. RBCs were stained with annexin V and PI after the infection of *A. hydrophila* at 6, 12 and 12 h, respectively. Green signal indicates early apoptosis cells and the red signal represents late apoptosis cells.

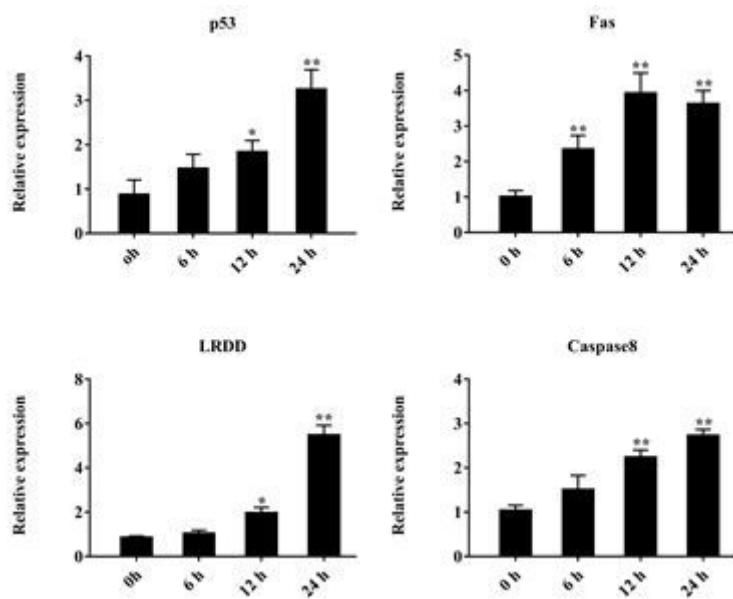
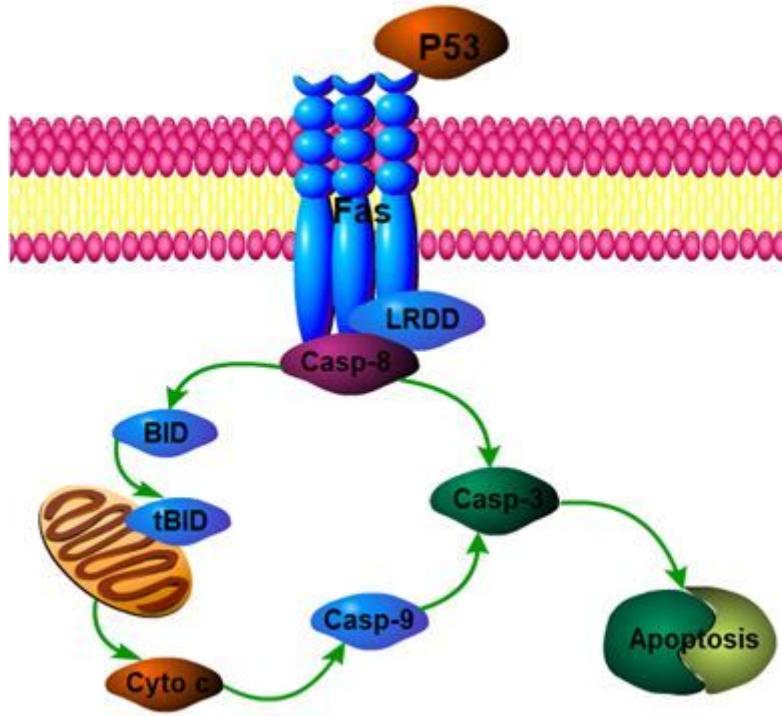


Figure 9

Validation of the transcriptomic DEGs using apoptosis-related genes of grass carp RBCs infected with *A. hydrophila* at 6 h, 12 h and 24 h post of the infection. Gene expression was analyzed by RT-qPCR, $2^{-\Delta\Delta Ct}$ method, using endogenous gene β -actin as a reference. Data represent means \pm SD. Statistical comparison of the mRNA levels detected at different time points was carried out by one way-analysis of variance (*P < 0.05, **P < 0.01).

**Figure 10**

A proposed apoptosis pathway model of grass carp RBCs induced by the infection of *A. hydrophila*.