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Myeloid differentiation factor 88 (Myd88) is involved in the innate immunity of black carp (*Mylopharyngodon piceus*) defense against pathogen infection

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ABSTRACT

Myeloid differentiation factor 88 (*MyD88*) is an important transduction protein in the Toll-like receptor signaling pathway. In this study, we identified the cDNA of the *MpMyD88* gene in black carp. We found that *MpMyD88* was widely distributed in the tissues tested and showed significant immune responses both *in vitro* and *in vivo* after stimulation with bacterial and pathogen-associated molecular patterns. After *MpMyD88* overexpression/silencing, proinflame-matory cytokines (TNF-α, IFN-α, IL-6, and IL-8) also showed significant up-regulation/down-regulation. Moreover, we found that the antibacterial ability of cells over-expressing *MpMyD88* was significantly stronger than that of control cells, while that of silenced *MpMyD88* was significantly lower than that in control cells. Besides, we found that the overexpression of *MpMyD88* significantly increased the activity of NF-κB. These results indicate that *MpMyD88* plays an important role in the innate immune response.

1. Introduction

The innate immune system is mediated by a pattern recognition receptor (PRR), that recognizes pathogen-associated molecular patterns (PAMPs), which causes the expression of effector molecules through signal transduction cascade effects [1]. Natural immunity plays an important role in lower vertebrates. Toll-like receptors (TLRs) are an innate immune recognition system of PAMPs [2]. Moreover, this activated signaling pathway can also regulate adaptive immune responses [3]. The myeloid differentiation factor 88 (MyD88) molecule is an important transduction protein in this signaling pathway and is a key target molecule for downstream signaling [4]. MyD88 was originally discovered in the differentiation of mouse myeloid cells and consists of 296 amino acid residues [5]. *MyD88* belongs to the Toll/IL-1R family and members of the death domain family, and its encoded protein has three functional regions: the N-terminal death domain, the intermediate region, and the C-terminal TIR domain [6].

In addition to TLR3, MyD88 mediates the activation of most TLRs [7,8]. The Toll-interleukin-1 (IL-1) receptor domain (TIR) of TLRs activates IL-1 receptor-associated kinases (IRAKs), such as IRAK1 and IRAK4, by recruiting MyD88 [9–11]. The interaction between activated IRAKs and Tumor necrosis factor receptor-associated factor 6 (TRAF6) initiates a cascade of signaling [12], while further activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) induce inflammatory responses [13–15]. Besides, MyD88 can also activate interferon regulatory factors (IRF-1, IRF-5, IRF-7), which play an important role in the induction of pro-inflammatory cytokines and interferons during host immune responses [12,16]. With the deepening study of TLRs and their signal transduction pathways, the *MyD88* gene of several species has been cloned and analyzed, including that of humans [17], mice [18], zebrafish [19], large yellow croaker [20], bastard halibut [21], and the Chinese scallop, *Chlamys farreri* [22]. However, the role of MyD88 in the immune response of the black carp remains unknown.

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In China, black carp is one of the “four famous domestic fishes” [23], and of the four domestic fishes, it is the most highly esteemed and the highest priced food fish, so that black carp is an important economic species for freshwater aquaculture in China. Black carp are affected by a large number of pathogenic microorganisms under natural conditions, especially *Aeromonas hydrophila*, which has caused serious economic losses to the black carp farming industry [24]. In the present study, we identified the full-length cDNA of *MpMyD88* in black carp and analyzed its distribution in healthy black carp. In addition, we studied the expression patterns of *A. hydrophila* and PAMPs at different times *in vivo* and *in vitro*. We also analyzed the effect of this gene on inflammatory factors and the antibacterial ability of black carp. Using the NF-κB luciferase plasmid, we demonstrated that this gene activates the NF-κB signaling pathway. Our research aims to provide a new perspective for a better understanding of the function of the *MpMyD88* gene in teleost fish and to better understand its role in the immune function in black carp.

2. Materials and methods

2.1. Ethics statement

All experiments with fish in this study were conducted in accordance with the guidelines on the care and use of animals for scientific purposes, set up by the Institutional Animal Care and Use Committee (IACUS) of Shanghai Ocean University, Shanghai, China. The IACUS approved this study within the project “Breeding of Black Carp” (approval number SHOU-16-014). The infection and dissection experiments were all performed under 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222) (Sigma, USA) anesthesia to minimize the suffering of fish.

2.2. Experimental fish, bacteria, cells

The black carp used in this experiment were reared by our research group in May 2017 and were fed at the No. 12 pond of Shanghai Ocean University Coastal Breeding Base. On April 3, 2018, we randomly selected 450 healthy black carp (mean ± SE weight 23 ± 2.8 g) for temporary feeding at the 3rd Gate Aquaculture System of Shanghai Ocean University. The water temperature was controlled at 28 °C ± 0.5 °C. During the temporary feeding period, the carp were fed and managed by scientific feeding methods [25], and, after two weeks, we carried out the subsequent experiments.

The *A. hydrophila* used in this experiment were obtained by members of the project group from the dying grass carp that suffered from bacterial septicemia [26,27]. After 16S rDNA sequencing comparison, it was found to be highly homologous to ATCC 7966 (Gen Bank Accession No. ASM1480v1) in the NCBI database. The strain was named AH10 in the laboratory and stored in the National Aquatic Animal Pathogen Bank (No. 2011AH10). Genomic information has been submitted to the NCBI database (accession number: CP011100.1).

The cell line used in this experiment was the black carp kidney cell line, MPK, provided by Professor Tiansheng Chen [28]. MPK was grown in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 100 units/ml penicillin and 100 mg/ml streptomycin, 0.055 mM sodium pyruvate. The cells were cultured in an incubator at 28 °C without carbon dioxide.

2.3. Black carp infection experiment and sample collection

We collect blood and 11 tissues (gill, liver, spleen, intestine, mid kidney, head kidney, heart, skin, fins, muscles, and brain) of 4 healthy black carps on ice for distribution analysis of *MpMyD88* (*Mylopharyngodon piceus* MyD88). Establishment of experimental groups and controls (60 healthy black carps per group) The fish in the control group were intraperitoneally injected with 100 μl phosphate-buffered saline (PBS), and those in the experimental group were intraperitoneally injected with 100 μl AH10 bacteria at a concentration of 4.2×10^7 CFU/ml (Semi-lethal concentration (LC50) determined in the pre-experiment). Immune tissues (gill, liver, spleen, intestine, mid kidney, and head kidney) were collected at 0, 4, 8, 12, 24, 48, 72 h and seven days after infection for the time-dependent expression profile analysis of *MpMyD88*. All samples were frozen in liquid nitrogen immediately after collection and then stored at −80 °C until total RNA was extracted.

2.4. Total RNA extraction and cDNA synthesis

The samples obtained were extracted with total RNA using Trizol reagent (Life Technologies, Darmstadt, Germany), and the total RNA concentration, purity, and quality were measured using 1% agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Nanodrop Technologies, USA). All RNA was stored at −80 °C until use. High Fidelity PrimeScript RT-PCR Kit (TaKaRa, Japan) was used, following the manufacturer's protocol to perform cDNA synthesis, with all cDNAs synthesized being stored at −20 °C.

2.5. Full-length clone of MpMyD88 cDNA

We identified a partial fragment of *MpMyD88* from a transcriptome library of black carp (accession number: SRP133863), and the specific primers used were *MpMyD88*-F1 and *MpMyD88*-R1 (Table 1). To obtain the full-length clone of *MpMyD88*, we used Rapid Amplification of cDNA Ends (RACE) technology to amplify the ends of the fragments. We used a SMARTer RACE 5′/3′ Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions to achieve end amplification with the primers listed in Table 1.

2.6. Bioinformatics analysis of MpMyD88

The nucleotide sequence homology of the *MpMyD88* gene was analyzed by the online software BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequence was predicted using the NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Protein domains were predicted using web-based CDD software (https://www.ncbi.nlm.ni-h.gov/Structure/cdd/wrpsb.cgi), and the signal peptide was analyzed by SignalP (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight and theoretical isoelectric point of *MpMyD88* were predicted by the ExPASy Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The three-dimensional structure was analyzed using SWISS-MODEL (http://swissmodel.expasy.org/). Multiple sequence alignments were performed with the amino acid sequences of MyD88 in other species using the BioEdit Sequence Alignment Editor. A phylogenetic tree was constructed based on the deduced amino acid sequence of the *MpMyD88* protein using MEGA 6.0 software and the neighbor-joining (NJ) ligation method.

2.7. Quantitative real-time PCR analysis

The cDNA samples were subjected to qPCR analysis after reverse transcription of RNA. The β-actin gene (GenBank accession No. AY289135) of black carp was used as a standard internal reference gene [29]. Specific primers for β-actin and *MpMyD88* are listed in Table 1. The 20 μl qPCR reaction system included 10 μl of 2 × TB Green Premix Ex Taq™, plus and minus primers containing 0.8 μl or 1.6 μl of cDNA, and 6.8 μl of double-distilled water. There were four replicates for each experimental group and an internal reference gene. The procedure for qPCR amplification was: 95 °C for 3 min, 95 °C for 5 s, 60 °C for 30 s cycle (repeated 40 times). Data analysis was performed using the $2^{-\Delta\Delta Ct}$ methods. Data from the control group and the experimental group were compared using one-way analysis of variance of SigmaPlot software.

Table 1
Sequences of primers used in this study.

Primer	Sequence (5′–3′)	Application
MpMyD88-F1	GCCGAAATGATGGACTTTACCT	Partial fragment amplification of *MpMyD88*
MpMyD88-R1	AATGTTCAGGGGAGTGCGAG	
MpMyD88-R2	CCGCAGCTGTCCACCACTGGAACCTG	5′RACE of *MpMyD88*
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
MpMyD88-R3	TCCATCATTTCGGCGACAGTCCTCCA	5′RACE of *MpMyD88*
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
MpMyD88-F2	GATGTAAGAGGATGGTGGTGGTC	3′RACE of *MpMyD88*
OUTER	TACCGTCGTTCCACTAGTGATTT	
MpMyD88-F3	CGCTCTAAACGCCTAATCCCTG	3′RACE of *MpMyD88*
INNER	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
MpMyD88-F4	CCAGGCACGTGTGTGTGGAC	RT-PCR of *MpMyD88*
MpMyD88-R4	CGAGCTCCTGGGCAAAGGCT	
TNFα-F	ACGCTGCCCTTACCGAGGGT	RT-PCR of *MpMyD88*
TNFα-R	AGGGCCACAGCCAGAAGAGC	
IFNα-F	ATGGCTCGGCCGATACAGGA	RT-PCR of *MpMyD88*
IFNα-R	TGGCATCCATGAGGCGGATGA	
IL6-F	TGCCGGTCAAATCCGCATGGA	RT-PCR of *MpMyD88*
IL6-R	CCCGGTGTCCACCCTTCCTCT	
IL8-F	CCTCACGGCGCGGGTTACAA	RT-PCR of *MpMyD88*
IL8-R	CCGCCGCAGGTTGTCAGGTG	
β-actinF	GGCACCGCTGCTTCCTCCTC	RT-PCR of *MpMyD88*
β-actinR	GCCTCTGGGCACCTGAACCT	
*MpMyD88*F-EcoR I	CCGGAATTCATGGCATCAAAGTCAAGTATAG	Recombinant plasmid
*MpMyD88*R-BamH I	CGCGGATCCCGGGGAGTGCGAGCGCC	

2.8. Plasmid construction and transfection

To construct the *MpMyD88*-overexpression vector, forward and reverse primers (Myd88-F1-EcoRI/Myd88-R1-BamHI) (Table 1) were designed using Primer 5.0 software to amplify the open reading frame (ORF) of *MpMyD88*. The pEGFP-N1 plasmid (Clontech, USA) and the PCR product were then digested with the corresponding restriction enzymes. The digested product was then purified and ligated to finally obtain a pEGFP-N1-*MpMyD88* recombinant plasmid.

For transfection, cells were seeded into six-well plates to achieve 70–80% confluence, washed twice with PBS and warmed with the antibiotic-free medium. Transient transfection was performed using Lipofectamine 3000 Reagent (Invitrogen, USA) according to the manufacturer's instructions. Subsequently, the original medium was replaced with complete medium, after 6 h transfection.

2.9. In vitro bacterial/virus PAMP stimulation

For *in vitro* studies, MPK cells (4×10^6 cells/ml final concentration) were incubated in six-well plates for 24 h, followed by purified flagellin from *Salmonella typhimurium* (FLA-ST) at a concentration of 10 ng/ml (purified flagellin from *Salmonella typhimurium*; Sigma-Aldrich), 10 μg/ml of lipopolysaccharide (LPS) (purified from *Escherichia coli*; Sigma-Aldrich), and 5 μg/ml of polyinosinic-polycytidylic acid (poly(I:C); Sigma–Aldrich)-stimulated MPK cells. The control groups were treated with PBS. To investigate the relevant expression profiles at different times, we treated the cells in 500 μl of TRIzol for 5 min at 0, 4, 8, 12, 16, 24, 30 and 36 h, respectively, and harvested the samples in parallel.

2.10. Overexpression/silencing of the MpMyD88 gene on the effects of A. hydrophila infection and pro-inflammatory cytokines

pEGFP-N1-*MpMyD88* and pEGFP-N1 were transfected into MPK cells by the method described in Section 2.8., as an experimental group and a control group, respectively, and each experiment was performed with six replicates [biological (i.e., independent) replicates]. Three of these groups were collected using TRIzol reagent (Life Technologies) after 24 h. Total RNA was reverse transcribed as described above (Section 2.4), and the expression of IFN-α (GenBank accession no: KR265208), IL-6, IL-8 [27], and TNF-α (GenBank accession no: KP192120.1) pro-inflammatory cytokines were detected by qPCR, the specific primers used were listed in Table 1. After 24 h, the experimental group and the control group were infected with *A. hydrophila* (2×10^6 cells) for 30 min, and then the cells were gently washed with PBS and incubated with fresh DMEM medium containing 100 μg/ml gentamicin for 1 h to remove extracellular bacteria. The cells were then incubated with 1% Triton X-100 for 15 min to lyse the cells and release live bacteria. Finally, the bacteria were diluted in a 10-fold gradient and incubated on an LB plate containing ampicillin for 16 h in a 28 °C incubator. Bacterial colonies were then counted and analyzed.

The small interfering siRNA of *MpMy88*, synthesized by the Shanghai Genepharma Company (Shanghai, China), was transfected into MPK cells (2×10^5/well) cultured in six-well plates. A negative control (NC) of siRNA was also transfected into cells. After 24 h, samples were collected by TRIzol reagent (Life Technologies). Total RNA was reverse transcribed as described in Section 2.4, and the expression of IFN-α, IL-6, IL-8, and TNF-α pro-inflammatory cytokines were detected by qPCR.

2.11. Dual-luciferase reporter assay

To test the effect of *MpMyD88* on the NF-kB pathway, we co-transfected MPK cells (3×10^5 cells) with 100 ng of NF-kB fluorescent plasmid (Promega, USA), 10 ng of pRL-TK vector (Promega, USA) and 100 ng of pEGFP-N1-*MpMyD88* or 100 ng of pEGFP-N1. After 24 h of transfection, cells were washed with PBS and lysed with lysis buffer (Promega, USA). The activity of firefly luciferase and *Renilla* luciferase was normalized using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. The results were calculated from three independent replicates.

2.12. Statistical analysis

Data from the real-time PCR were expressed as the means ± SE. All experimental data were analyzed by one-way ANOVA using SPSS 18 analysis software (IBM, Armonk, NY, USA). Statistically, $P < 0.05$ was considered to be significant.

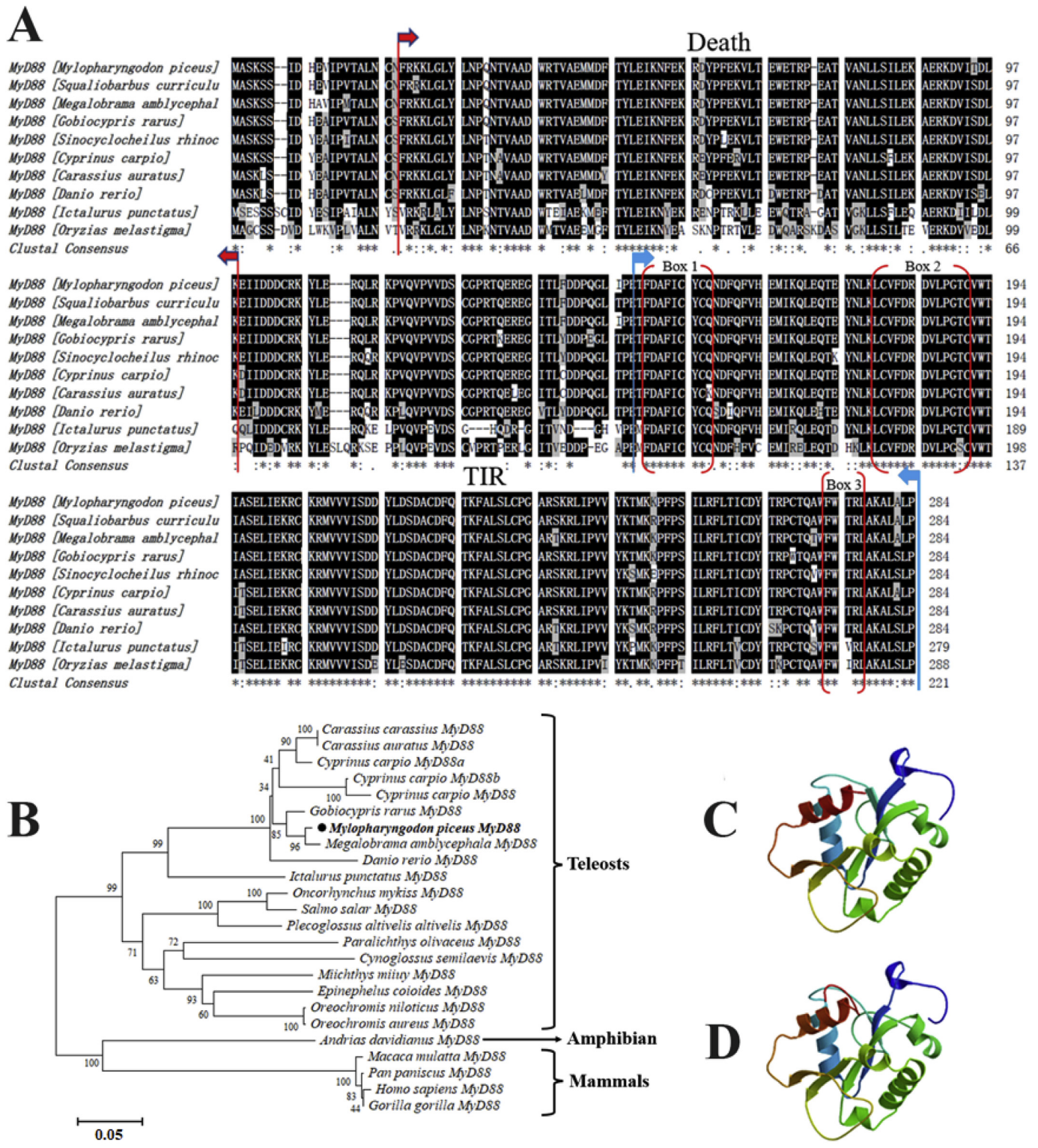

Fig. 1. Bioinformatics analysis of *MpMyD88*. (A) Multiple alignment analysis of *MpMyD88* and other known MyD88 proteins. The region indicated by the red arrow is the N-terminal death domain, and the region indicated by the blue arrow is the C-terminal TIR domain. Three conserved motifs, Box 1, Box 2, and Box 3, are framed. Identical (*) and similar (. and) residues identified by the BioEdit program are indicated. (B) Phylogenetic tree showing homology between *MpMyD88* and *MyD88* genes from other species. The tree was constructed by a BioEdit-generated multiple sequence alignment of amino acid sequences using the neighbor-joining method in MEGA 6. The topological stability of neighbor-joining trees was evaluated by 10,000 bootstrap replications. Numbers at branches indicate bootstrap values (%). (C) The predicted three-dimensional structure of the *MpMyD88* TIR domain (134 aa to 284 aa) and the solution structure of the TIR domain of human MyD88 (SMTL id: 2js7.1). (D) Three-dimensional structure of the TIR domain of human MyD88. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. cDNA cloning and sequence analysis

The full-length cDNA of 1658 bp *MpMyD88* (GenBank accession no: MH898877) was successfully cloned by RACE technology. The 5′-untranslated region (UTR) had 110 bp, the 3′-UTR had 693 bp, the ORF had 855 bp, and the encoded protein consisted of 284 amino acids. No signal peptide was found, and its theoretical isoelectric point and molecular mass were 5.66 and 33.02 kDa, respectively. The death (Phe21–Lys98) and TIR (Thr148–Pro284) domains are marked with arrows in Fig. 1A. We found three highly conserved boxes (Box 1: ^{149}FDAFICYCQ^{157}, Box 2: ^{179}LCVFDRDVLPGTC^{191}, and Box 3: ^{273}FWTRL^{277}) in the TIR domain (Fig. 1A). The similarity of the amino acid sequence of MyD88 between black carp and other teleost fish is about 60–99%. Among them, *MpMyD88* shares the highest similarity with *Megalobrama amblycephala* MyD88 sequences(Fig. 1B). Phylogenetic analysis was conducted using the Neighbor-joining method based on MyD88 sequences retrieved from NCBI database (Accession numbers used in this study are given in Suppl. Table 1). The MyD88 analyses revealed monophyletic teleost and cyprinid clusters, with *MpMyD88* very closely related to homologs from other cyprinids (Fig. 1B). The 3D model predicted by SWISS-MODEL showed that the TIR domain of *MpMyD88* consisted of a five-strand parallel β-shell surrounded by six α-helixes that formed a global fold (Fig. 1C), which was similar to the structure of the human MyD88 (Fig. 1D). Our data suggest that *MpMyD88* may function similarly in fish as it does in mammals.

3.2. qPCR analysis of the expression of MpMyD88 mRNA in healthy and A. hydrophila-infected tissues

To investigate the expression of *MpMyD88* in 11 tissues and blood, specific primers were used for qPCR, and the results are shown in Fig. 2A. *MpMyD88* was widely expressed in various tissues and had the highest expression in liver, and high expression in gill, fin ray, and spleen, with the weakest expression being in the intestines and blood.

The changes in *MpMyD88* expression in the immune tissues (gill, liver, spleen, intestines, mid kidney, and head kidney) of the black carp after infection with *A. hydrophila* are shown in Fig. 2B. The expression of *MpMyD88* in the six immune tissues tended to decrease first and then increase. The expression level decreased significantly at 4 h after infection, with the expression level reaching a peak at 8 h (spleen, intestines, and mid kidney) or 12 h (gill, liver, and head kidney), and the expression level returned to a normal pre-infection value on day 7.

3.3. Expression of MpMyD88 after FLA-ST, LPS, and poly(I:C) stimulation in vitro

After the MPK cells were stimulated by FLA-ST, LPS, or poly(I:C), the expression of *MpMyD88* mRNA was analyzed by qPCR (Fig. 3). Overall, all three stimuli resulted in a significant up-regulation of *MpMyD88*, and peaked at 24 h, which was 120.10-fold (Figs. 3A), 45.96-fold (Figs. 3B), and 36.78-fold (Fig. 3C) for FLA-ST, LPS, or poly (I:C), respectively. The expression of *MpMyD88* in response to the three different stimuli basically followed the trend associated with infection, by decreasing first and then rising.

3.4. Over-expression/silencing of the MpMyD88 gene on the effects of pro-inflammatory cytokines

To further investigate the role of *MpMyD88* in the black carp immune system, we overexpressed and silenced expression of the *MpMyD88* gene in MPK by pEGFP and siRNA techniques, respectively. Fig. 4A and B shows the efficiencies of *MpMyD88* overexpression and silencing, which were up-regulated 358.11-fold and down-regulated 2.12-fold, respectively. We then analyzed the expression profiles of TNF-α, IFN-α, IL-6, and IL-8 genes by qPCR. After overexpression of *MpMyD88*, TNF-α, IFN-α, and IL-6 increased significantly, but IL-8 increased only slightly, as shown in Fig. 4C. For the silencing, TNF-α, IFN-α, and IL-6 were significantly decreased, and IL-8 was also successfully inhibited (Fig. 4D).

To evaluate the effect of *MpMyD88* overexpression/silencing on the ability of MPK cells to resist bacteria, intracellular *A. hydrophila* cells were collected from the inoculated fish according to the method of section 2.10, and the count results after 16 h are shown in Fig. 5A. The data show that *A. hydrophila* was significantly less invasive in cells transfected with pEGFP-N1-*MpMyD88* (90.4% reduction) than in the control group. The data in Fig. 5B shows that A. hydrophila in the cells transfected with si-*MpMyD88* was significantly higher (175.46% reduction) than that of the control group.

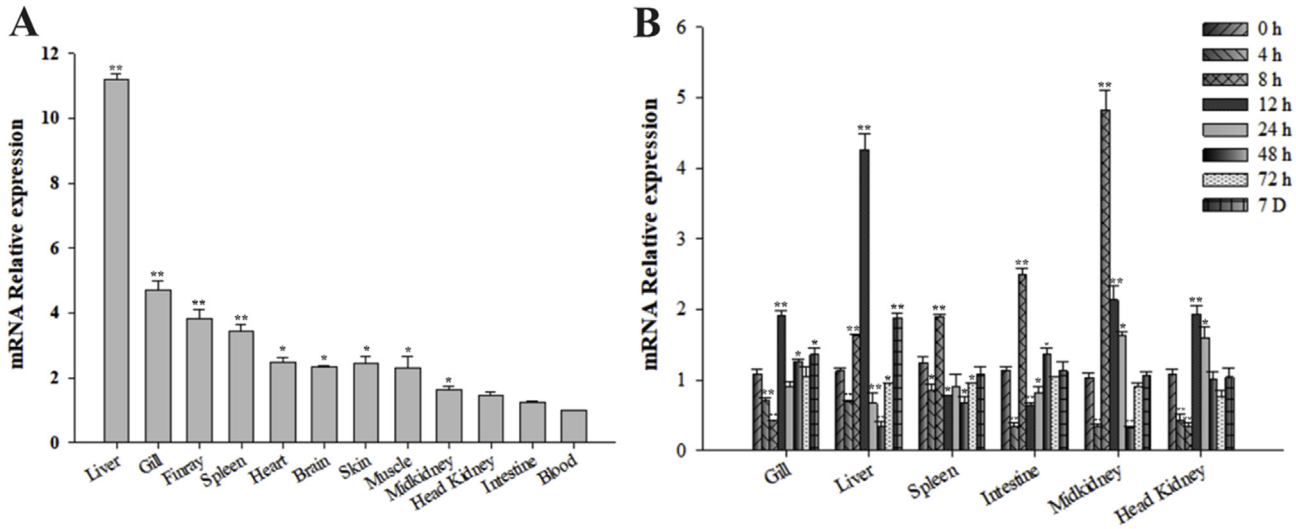

Fig. 2. (A) Tissue expression of the *MpMyD88* gene in twelve healthy tissues of black carp determined by qPCR. Gene expression levels of all tissues are expressed relative to that of blood. (B) Expression of the *MpMyD88* gene in immune tissues after *A. hydrophila* infection. The data of Fig. 2 were calculated and normalized relative to the expression of beta-actin mRNA levels. Data represent the mean ± SE of individual RNA samples of four fish (n = 3). Asterisks indicate a statistically significant difference compared to uninfected controls: *P < 0.05 and **P < 0.01.

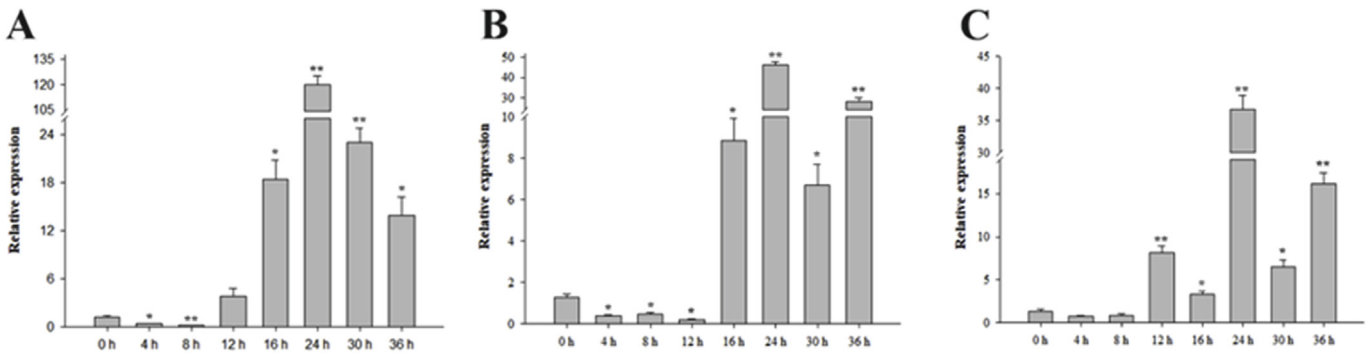

Fig. 3. Expression of the *MpMyD88* gene after stimulation with FLA-ST (A), LPS (B), and poly (I: C). Relative expression was calculated and normalized according to β-actin mRNA expression. Data represent mean ± SE. Asterisks indicate a statistically significant difference compared to controls: *P < 0.05 and **P < 0.01.

3.5. NF-κB activation in pEGFP-N1-MpMyD88 co-transfected MPK cells

We investigated the involvement of *MpMyD88* in NF-κB activation by co-transfection of MPK cells with NF-κB luciferase plasmid, pRL-TK vector, and pEGFP-N1-*MpMyD88* or pEGFP-N1. As shown in Fig. 6, overexpression of the *MpMyD88* gene significantly increased the activity of NF-κB.

4. Discussion

MyD88 has been identified in a range of different animals and has been extensively studied from vertebrates to invertebrates [30]. In this study, we successfully cloned the full-length *MpMyD88* cDNA of the black carp, which was 1658 bp in length and contained an 855 bp ORF encoding a protein of 284 amino acids. The deduced protein contained an N-terminal death domain and the typical domain structure of the C-

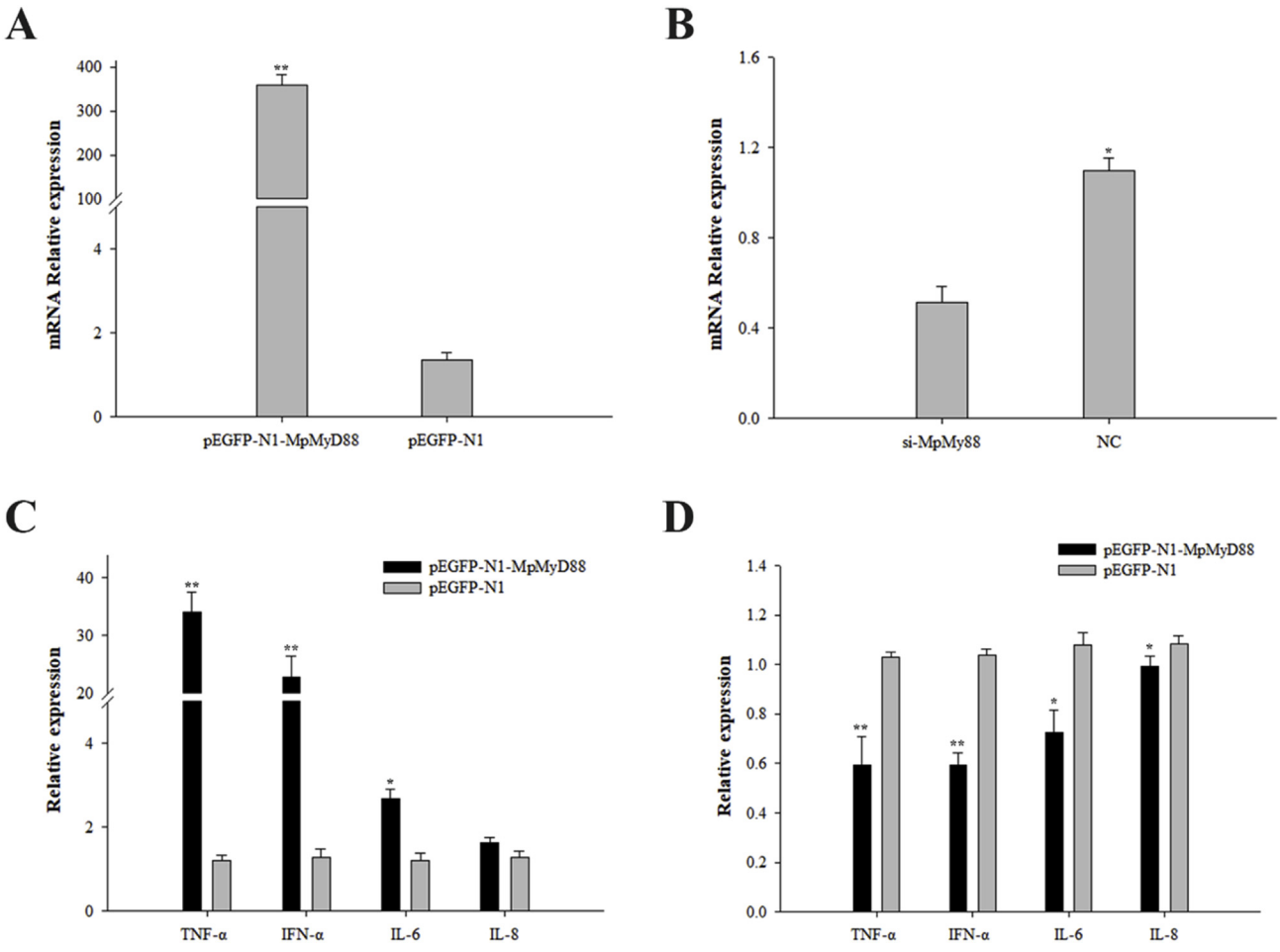

Fig. 4. (A) The over-efficiency of the pEGFP-N1-*MpMyD88* plasmid after transfection of MPK cells for 24 h pEGFP-N1 is the control group. (B) The silencing efficiency of the plasmid after transfection of MPK cells for 24 h. (C) Profiles of immune-associated molecule expression in MPK cells transfected with pEGFP-N1-*MpMyD88*. MPK cells were transfected with pEGFP-N1-*MpMyD88* or pEGFP-N1 vectors and expression of immune-associated molecules (TNF-α, IFN-α, IL-6, and IL-8) were compared with that in the control groups. (D) Profiles of immune-associated molecule expression in MPK cells transfected with si-*MpMyD88*. MPK cells were transfected with si-*MpMyD88* or NC and expression of immune-associated molecules (TNF-α, IFN-α, IL-6, and IL-8) was compared with that in the control groups. The data in Fig. 4 represents the mean ± SE. Asterisks indicate a statistically significant difference compared to controls: *P < 0.05 and **P < 0.01.

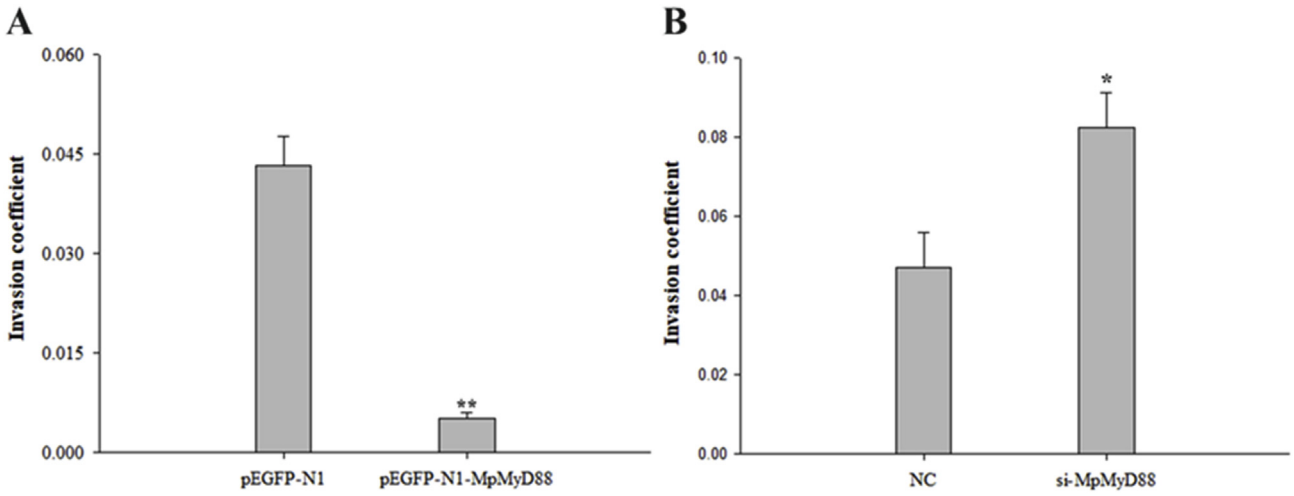

Fig. 5. (A) Effect of *MpMyD88* over-expression on *A. hydrophila* invasion. MPK cells were transfected with pEGFP-N1-*MpMyD88* or pEGFP-N1 vector for 24 h prior to infection with *A. hydrophila*. (B) Effect of *MpMyD88* silencing on *A. hydrophila* invasion. MPK cells were transfected with si-*MpMyD88* or NC for 24 h prior to infection with *A. hydrophila*. In Fig. 5, the invasion coefficient was calculated as the ratio of the number of intracellular bacteria to the number of bacteria applied. Data represent the mean ± SE (n = 4). Asterisks indicate a statistically significant difference compared to controls: *P < 0.05 and **P < 0.01.

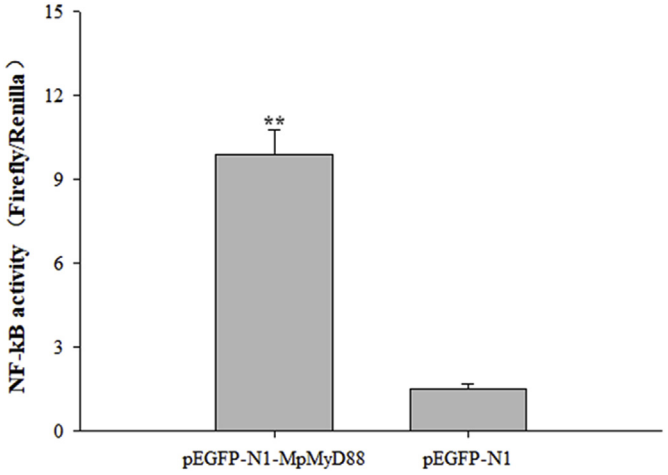

Fig. 6. Reporter gene activation by *MpMyD88* transfection. MPK cells were co-transfected with an NF-κB Luc reporter plasmid, pRL-TK vector, and plasmid pEGFP-N1-*MpMyD88* or pEGFP-N1 vector. Data represent mean ± SE of the ratio of firefly to Renilla luciferase fluorescence. Asterisks indicate a statistically significant difference compared to controls: *P < 0.05 and **P < 0.01.

terminal TIR domain. In addition, three highly conserved regions, Box 1, Box 2, and Box 3, similar to those found in mammals, were found in the TIR domain, consistent with MyD88 proteins from other species [18,19,31]. In mammals, it was originally suggested that the interaction between MyD88 and TLR relies on the binding of the TIR domain in the TLR to the TIR domain of the MyD88 protein. The TIR domain of the *MpMyD88* protein is highly conserved from fish to mammals during evolution, indicating a functional similarity of the *MpMyD88* protein in TLR signaling [32]. Box 1 and Box 2, which are conserved in the TIR domain, are crucial for MyD88 to interact with TLR or IL-1R to regulate downstream elements [33]. The main function of the death domain is to recruit IRAKs, such as IRAK1, IRAK2, and IRAK4, etc. [34–36], which play an important role in death signal transduction, induction of apoptosis, and inflammatory response [37]. In our 3D model re-constructing the *MpMyD88* protein, the domains of black carp and human MyD88 were very similar, again indicating that *MpMyD88* may function similarly in fish as in mammals.

By analyzing the phylogenetic tree, we found that black carp were first clustered with *Megalobrama amblycephala*, with the teleosts being clearly clustered together. MyD88 phylogenetic analysis supports traditional taxonomy, which means that intergeneric gene homology seems to establish more intimate associations. This association is confirmed by a high degree of similarity in the amino acid sequence among the teleost fish as determined by sequence alignment. This phenomenon is consistent with the concept that biological evolution records the genetics and variation of biology, from morphological features to genetic sequences [38].

The expression of *MyD88* has been studied in many species, although the expression profiles vary from species to species. In atlantic salmon, MyD88 is highly expressed in the liver and gill [39]. In the large yellow croaker, *MyD88* was most expressed in the spleen but was found to be most weakly expressed in the back muscles [20], whereas, in the miiuy croaker, *MyD88* was expressed mostly in the liver and the gill, but the expression was relatively low in the kidney and spleen [32]. In the present study, expression of *MpMyD88* was detected in all tissues examined in the black carp. The highest expression was in the liver, followed by the gill (Fig. 2A), findings which were similar to the studies by Pawapol et al. [40], and Whang et al. [41]. These reports show that *MpMyD88* is normally expressed at high levels in liver tissue and is an important immune organ in teleost fish, suggesting that MyD88 plays an important role in the immune defense system of teleost fish [42]. In addition, *MpMyD88* is highly expressed in the gill of the black carp, an organ that is easily invaded by pathogens. Gill is important immune tissues for teleost fish, and many studies have shown that many immune genes are highly expressed in the gill of bony fish, such as TLR and IFN [43,44]. High expression of *MpMyD88* in gill suggests that this molecule may play a role in the regulation of mucosal immunity and participate in innate responses to pathogens through the TLR signaling pathway.

A large body of data indicates that activation of MyD88 plays an important role in the innate immune system. Studies by Deguine et al. show that MyD88 plays a key role in TLR-induced inflammatory processes in mammals [45]. Studies in the challenge of fish infection have shown that the expression of *MyD88* was associated with bacterial and LPS stimulation, although there were differences in the expression profiles of different fish [32,46,47]. In this study, we studied the expression level of *MyD88* in immune-related tissues after *A. hydrophila* infection (Fig. 2B). The data in the present study showed that, after infection with *A. hydrophila*, the immune tissues sampled were found to exhibit high levels of *MpMyD88* expression at the early stages of infection. This indicates that MyD88 plays a central role in the antibacterial immune response of teleost fish [19]. In our study, we found

that the expression profile of *MpMyD88* was broadly similar to that in the immune tissues of black carp after infection with *A. hydrophila*. Initially, infected fish exhibited a downward trend in *MpMyD88* expression, after which *MpMyD88* expression was significantly up-regulated, reaching peak expression 8–12 h after infection and returning to normal levels of expression after 72 h of infection. This is similar to the studies by Yao et al. [20], Huang et al. [48], and Zou et al. [49]. We hypothesize that the sharp down-regulation of *MpMyD88* expression in immune tissues may be due to the recruitment of cells expressing *MpMyD88* at the lesion site. The slow recovery followed by significant up-regulation of *MpMyD88* expression may be due to the up-regulation of gene expression, proliferation, or recruitment of *MpMyD88*-expressing cells in tissues. Since MyD88 is a transduction protein in multiple signaling pathways [4,50], it is difficult to predict its involvement in immune response and further research is needed to better understand the mechanisms by which *MyD88* expression is regulated.

LPS and flagellin (FLA-ST) are major components of Gram-negative bacteria, which activate the NF-κB pathway and induce the involvement of pro-inflammatory cytokines in immune defense mechanisms [51,52]. Poly(I:C) is a viral double-stranded RNA analog recognized by TLR3 that is involved in transduction of signals to activate the innate immune system to produce key pro-inflammatory cytokines and chemokines [53]. In this study, the expression of the *MyD88* gene was detected in black carp injected with *A. hydrophila*; as a consequence, we expected to be able to detect the *in vitro* expression of *MpMyD88* in MPK cells stimulated by bacterial and viral PAMP (flagellin, LPS, and poly (I:C)). Our results showed that *MpMyD88* gene expression was significantly up-regulated by FLA-ST and LPS stimulation and peaked at 24 h. The results were similar to the results of Whang et al. [41]. MyD88 is involved in TLR signaling and plays an important role in infection-induced blood cell activation and cytokines [54]. In our results, under the stimulation of poly(I:C), *MpMyD88* expression was also significantly up-regulated and peaked at 24 h, similar to the results from *Trachinotus ovatus* [55], *Paralichthys olivaceus* [21], and *Ctenopharyngodon idella* [56]. Studies have shown that the secretion of NO_2 in macrophages is severely impaired after treatment with poly(I:C) in MyD88-deficient macrophages [57]. In addition, the overexpression of *MyD88* in flounder resulted in a significant decrease in the inhibition of viral replication by poly(I:C), but a significant increase in the inactivation of MyD88 [58]. Studies have shown that TLR3 and cell surface TLR22 in teleost fish can recognize poly(I:C) [59]. Johnson et al. indicated that MyD88 negatively regulated TLR3-induced corneal inflammation in a c-Jun N-terminal kinase-dependent manner [60]. A report by Siednienko et al. also showed that TLR3-mediated IFN-β is induced and negatively regulated by the TLR adaptor MyD88 adaptor-like [61]. In our study, MPK cells were significantly down-regulated in the early stage of *MpMyD88* induction by poly(I:C), which may be related to the negative regulation by TLR3. Our results showed that *MpMyD88* plays an important role in the immune response of black carp to Gram-negative bacteria or viruses. However, further experiments are needed to clarify the negative regulatory mechanisms involved.

There are many reports that the TLR signaling pathway in immune tissues is significantly activated after *A. hydrophila* invades fish [27,62,63], indicating that the TLR signaling pathway plays an important role in the response of fish to pathogen challenge [64,65]. In addition, MyD88 is the downstream signal transduction adapter for almost all TLRs, so MyD88 in fish plays a key role in the immune system of the fish. The effect of *MpMyD88* on *A. hydrophila* invasion was analyzed in our study by the overexpression/silencing of *MpMyD88* in MPK cells. We found that overexpression of *MpMyD88* is effective in preventing bacterial invasion (Fig. 5A), while Fig. 5B shows that MPK is significantly reduced in resistance to pathogens after the *MpMyD88* knockdown. Our results again indicated that *MpMyD88* is involved in the immune response of black carp against pathogen infection. Based on the immune mechanism of black carp infected by *A. hydrophila* [27], we studied the expression of four immune-related genes (TNF-α, IFN-α, IL-6, and IL-8), which play an important role in the inflammatory response and immune system [64–67]. In our results, in response to overexpression of *MpMyD88* in MPK, the four immune genes were significantly up-regulated, except for IL-8 (Fig. 4C), while the four immune genes were significantly down-regulated after knockdown of *MpMyD88* (Fig. 4D). The results showed that *MpMyD88* is involved in the immune and inflammatory responses of MPK cells and may also play a very important role. In addition, related studies have shown that NF-κB and AP-1 can induce the expression of pro-inflammatory cytokines [68,69]. Studies have shown that mammalian MyD88 can activate the NF-κB signaling cascade. In human Huh7 hepatoma cells, expression of the full-length human MyD88 resulted in activation of NF-κB activity and its N-terminal region polypeptide (amino acids 1–151) was still capable of inducing NF-κB activity. Conversely, the dominant-negative mutant of MyD88 (amino acids 152–296) did not induce NF-κB activity [70]. Recent reports indicated that the MyD88 of fish could induce the activity of NF-κB, confirming the functional conservation of MyD88 during the evolution of vertebrate innate immunity from fish to mammals [31,49,71]. In this study, overexpression of the *MpMyD88* gene significantly activated the NF-κB signaling pathway and inflammatory factors(TNF-α, IFN-α, IL-6, and IL-8)in MPK cells. These data are consistent with previous studies of MyD88 from different species and indicate *MpMyD88* in black carp may play similar roles in triggering the host NF-κB signaling cascade resulting in proinflammatory cytokine induction and elimination of invading microorganisms.

In summary, we successfully identified the *MpMyD88* gene in black carp, and our study showed that *MpMyD88* is involved in the innate immunity of the black carp to resist pathogens. *MpMyD88* was widely expressed in various tissues of healthy fish, in which high levels of expression are detected in liver, gill, fin, and spleen, with low levels of expression being detected in blood and intestines. In addition, after infecting black carp with *A. hydrophila in vivo*, it was found that *MpMyD88* was significantly up-regulated in the immune tissues that were assessed. With regards to *in vitro* studies, we stimulated MPK cells with three kinds of PAMPs (flagellin, LPS, and poly(I:C)), and found that *MpMyD88* showed extremely significant up-regulation, indicating that the transcriptional expression of *MpMyD88* plays an important role in the immune response against pathogens in black carp. Our studies indicate that overexpression of *MpMyD88* inhibited the expansion of *A. hydrophila* in MPK cells, that the NF-κB signaling pathway was significantly activated and that TNF-α, IFN-α, and IL-6 were significantly up-regulated. Besides, after knockdown of *MpMyD88*, the ability of MPK cells to inhibit *A. hydrophila* multiplication decreased significantly, and we found that TNF-α, IFN-α, IL-6, and IL-8 were significantly down-regulated. These results indicate that *MpMyD88* can participate in the immune defense mechanism against pathogens in black carp by activating the expression mechanisms of other genes. *MpMyD88* plays an important role in the innate immunity of black carp against pathogens. However, the role of *MpMyD88* in immune regulation needs further research to better understand the disease-resistance mechanism of fish, to determine new disease control strategies based on drug intervention, and to provide some guidance for the cultivation of new disease-resistant varieties of black carp.

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Appendix A. Supplementary data

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