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A novel NK-lysin in hybrid crucian carp can exhibit cytotoxic activity in fish cells and confer protection against *Aeromonas hydrophila* infection in comparison with *Carassius cuvieri* and *Carassius auratus* red var

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ABSTRACT

NK-lysin, an effector of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), not only exhibits cytotoxic effect in fish cells, but also participates in the immune defense against pathogenic infection. In this study, ORF sequences of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin were 369 bp. Tissue-specific analysis revealed that the highest expressions of RCC-NK-lysin and WCC-NK-lysin were observed in gill, while the peaked level of WR-NK-lysin mRNA was observed in spleen. *A. hydrophila* infection sharply increased RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA expression in liver, trunk kidney and spleen. In addition, elevated levels of NK-lysin mRNA were observed in cultured fin cell lines of red crucian carp (RCC), white crucian carp (WCC) and their hybrid offspring (WR) after Lipopolysaccharide (LPS) challenge. RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin exerted regulatory roles in inducing ROS generation, modulating mitochondrial membrane potential, decreasing fish cell viability and antagonizing survival signalings, respectively. RCC/WCC/WR-NK-lysin-overexpressing fish could up-regulate expressions of inflammatory cytokines and decrease bacterial loads in spleen. These results indicated that NK-lysin in hybrid fish contained close sequence similarity to those of its parents, possessing the capacities of cytotoxicity and immune defense against bacterial infection.

1. Introduction

Evidences are emerging that the exposure to ambient stressors may trigger the occurrences of severe diseases [1]. In general, biotic or abiotic stressors can lead to physiological malfunction in fish [2]. Ecological deterioration and environmental pollution can suppress fish immunity, thus rendering fish less resistant against pathogenic infection [3]. Cell killing mechanism plays a pivotal role in immune defense against invading pathogens and exhibits a cytotoxic effect on infected tissues or cells [4]. Antimicrobial peptides (AMPs) are a diverse class of peptides with small molecular weight and either a linear or cyclic structure [5,6], which can serve as immunoregulatory molecules capable of defending against pathogenic invasion within the host [7,8]. In general, cationic AMPs can electrostatically interact with the surface of negative-charged cell membranes [9], whereas some AMPs may disrupt cell membranes by targeting to some specific membrane compounds [10,11]. In addition, AMPs usually exhibit broad-spectrum activity against microorganisms and their microbial targets may rarely develop resistant phenotypes [12].

As is well known, NK cells and CTLs are important immune cell components in the processes of host defense against a broad range of pathogenic invasion [13]. Upon stimulation, cytolytic granules derived from NK cells and CTLs can release cell-lytic molecules, including granzymes, perforin and granulysin. NK-lysin is firstly discovered from pig intestine, which is a member of saposin-like protein (SAPLIP) superfamily and shows a structural homologue to granulysin [14]. Additionally, NK-lysin is a novel effector secreted by CTLs and NK cells, playing a vital role in the innate immune defense against infectious

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pathogens [15,16]. Emerging evidences have showed that the bactericidal activity and tumourolytic effect of NK-lysin may require intact disulfides formed by conserved cysteine residues [17,18].

In contrast to innate immunity of invertebrate, fish not only possess various forms of pathogen-recognizing properties, but also contain a developed complement system and apoptosis-related pathways [19,20]. Despite the mammalian AMPs have been extensively researched, only a few studies focus on the function of teleostean AMPs, especially NK-lysin. In recent years, architecture and expression patterns of teleostean NK-lysins are identified in *Ictalurus punctatus* [21], *Cynoglossus semilaevis* [22], *Oreochromis niloticus* [23] and *Boleophthalmus pectinirostris* [24], but the data on comparative analysis of immunoregulatory function of chimeric NK-lysin from hybrid offspring with those of its parents are sparse.

Hybridization may give rise to species with novel capabilities and chimeric genes formed by fusion of various genomes may lead to changes of gene structure and occurrence of nonsynonymous variants, thus affecting downstream signalings upon stimulation [25–27]. In fish, recent findings imply that hybrid offspring can exhibit a low susceptibility to pathogenic infection compared with those of its parental species [28,29]. Crucian carp (*Carassius auratus*) is one of the most important economic freshwater fish and abundant in lakes, rivers and reservoirs in China, which is popular with fish farmers [30]. Currently, white crucian carp (*Carassius cuvieri*, WCC) and red crucian carp (*Carassius auratus* red var, RCC) are classified into different species in the genus of *Carassius* [31], thus generation of hybrid crucian carp (WR) by crossing of WCC (2n = 100, ♀) and RCC (2n = 100, ♂) is considered as interspecific hybridization [32]. However, occurrence of global climate anomaly may promote a lingering effect in expansion of water-borne pathogenic diseases, threatening the survival of aquatic animals [33].

In this study, the aims were to study architectures of RCC/WCC/WR-NK-lysin and measure their expression patterns upon *in vitro* stimuli. To further characterize their functions, we also assessed the reporter genes and immune-related signalings, which may provide a new insight into the immune regulation of hybrid fish and its parents.

2. Materials and methods

2.1. Animals

Hybrid fish (WR) were generated by crossing white crucian carp (*Carassius cuvieri*, WCC, ♀) and red crucian carp (*Carassius auratus* red var, RCC, ♂) [34]. RCCs, WCCs and WRs (average length 13.2 ± 0.52 cm) were obtained from an aquaculture base in Wang Cheng district (Changsha, China), which were acclimatized for two weeks and fed with commercial diet twice daily till 24 h before challenge experiment, respectively.

2.2. Gene cloning and bioinformatics analysis

According to the high-conserved domains of NK-lysin sequence in Genbank database, touchdown PCR assay was performed to obtain ORF sequences of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin, respectively [35]. Gene-specific primers were shown in Table 1. Based on our previous studies, domain alignment and structure prediction of NK-lysin were constructed by NCBI Blast program, motif scan, ExPASy, SignalP 5.0, PredictProtein and Phyre 2 server, respectively. In addition, phylogenetic tree analysis was constructed by MEGA 6.0, using the neighbor-joining method with 1000 bootstrap replications [36].

2.3. Immune challenge with A. Hydrophila

A. hydrophila was cultured for 24 h at 28 °C, centrifuged at 10,000×*g* for 15 min at 4 °C, and resuspended in 1 × PBS (pH 7.3) [37]. Immune challenge was performed by intraperitoneal injection of 100 μl suspension of *A. hydrophila* (1×10^7 CFU ml^{-1}) in PBS. In addition, RCCs, WCCs and WRs injected with 100 μl sterile PBS were used as the controls. Each group was composed of six fish and each treatment contained three replicates under the same conditions. Tissues were isolated at 0, 6, 12, 24, 36 and 48 h post-infection, immediately frozen in liquid nitrogen and preserved in −80 °C.

2.4. Cell culture

RCC fin cells (RCCFCs), WCC fin cells (WCCFCs), hybrid crucian carp fin cells (WRFCs) and *Epithelioma papulosum cyprinid* (EPC) were cultured at 26 °C with a humidified atmosphere of 5% CO_2 [38,39].

2.5. LPS treatment

RCCFCs, WCCFCs and WRFCs were seeded in 6-well plates at 80% confluence for 18 h. Then, cell culture medium was replaced with fresh medium containing 500 ng/ml or 1000 ng/ml of LPS (from *Escherichia coli* O111:B4, Sigma, USA) [40,41]. After that, cells were harvested at 0, 6, 12, 24, 36 and 48 h post-treatment, immediately frozen in liquid nitrogen and preserved in −80 °C.

Table 1
The primer sequences used in this study.

Primer names	Sequence direction (5' → 3')	Use
NK-lysin-F	ATGCTGCGGAGAATCGTC	clone
NK-lysin –R	CTATTTCTTGCAAACACC	clone
pc-NK-lysin-F	CGGGGTACCCCATGCTTCACTGGGAAATGCAC	vector
pc-NK-lysin-R	CCGGAATTCTTACTTGTCGTCATCGTCTTTGTAGTCTTTCTTGCAAACACCAAT	vector
RT-18 S–F	CCGACCCTCCCTCACG	qPCR
RT-18 S-R	GCCTGCTGCCTTCCTTG	qPCR
RT-NK-lysin-F	TGCGGAGAATCGTCGTG	qPCR
RT-NK-lysin-R	GGTTTTGGCGTCATCAGTAG	qPCR
RT-IL-1beta-1-F	CCTGACAGTGCTGGCTTTG	qPCR
RT- IL-1beta-1-R	AATGATGATGTTCACCACCTTC	qPCR
RT-IL-1beta-2-F	TCTTCGCATCCTCACAGCAT	qPCR
RT-IL-1beta-2-R	CAGCGTCACAGCCTTCAAAT	qPCR
RT-TNFalpha-1-F	GGATTGCTGCCCTCACGG	qPCR
RT-TNFalpha-1-R	CTTTGGACACTTTAGGTTCATACG	qPCR
RT-TNFalpha-2-F	GTGGGGTCCTGCTGGCT	qPCR
RT-TNFalpha-2-R	CTGGTCCTGGTTCTGTTTC	qPCR
RT-hlyA-F	GGCCGGTGGCCCGAAGATACGGG	qPCR
RT-hlyA-R	GGCGGCGCCGGACGAGACGGGG	qPCR
RT-GAPDH-F	CAGGGTGGTGCCAAGCG	qPCR
RT-GAPDH-R	GGGGAGCCAAGCAGTTAGTG	qPCR

2.6. RNA isolation, cDNA synthesis and qRT-PCR assay

Total RNA was extracted from isolated tissue and harvested cell samples by using HiPure Total RNA Mini kit (Magen, China). Then, concentration and integrity of total RNA were determined by measurement of 260/280 nm absorbance and agarose gel electrophoresis, respectively [42]. 1000 ng of purified total RNA was used for cDNA synthesis using Revert AidTM M-MuLV Reverse Transcriptase Kit (MBI Fermentas, USA). To investigate the transcript changes of immune-related genes, qRT-PCR assay was performed as previously described [43]. 18 S rRNA (XR_003291850.1) was used as internal control [44]. Primers used in this study were shown in Table 1. Primer specificity was confirmed and each sample was analyzed in triplicate. At the end of qRT-PCR assay, melting curve analysis was implemented to confirm credibility of each qRT-PCR analysis. Results were measured by using Applied Biosystems QuantStudio 5 Real-Time PCR System with $2^{-\triangle\triangle Ct}$ methods [45].

2.7. Plasmid construction

The above ORF sequences of RCC-NK-lysin, WCC-lysin and WR-NK-lysin were ligated to pcDNA3.1 plasmid, which were subjected to sequencing confirmation as previously described [46]. Then, endotoxin-free plasmids were prepared by using Endo-free plasmid kit (Omega).

2.8. Western blotting

EPC cells were seeded in 6-well plates and incubated for 24 h. Cells were transfected with pcDNA3.1, pcDNA3.1-RCC-NK-lysin, pcDNA3.1-WCC-NK-lysin and pcDNA3.1-WR-NKlysin plasmid by using Lipofectamine 3000 reagent and then cultured with fresh medium, respectively. After that, cells were harvested at 48 h post-transfection and lysed by RIPA solution. Protein concentration in supernatants of cell lysate samples were determined by BCA method. In brief, western blotting assay was performed as described previously [47,48]. Following antibody incubation, PVDF membranes were washed in TBST and visualized. In addition, anti-β-actin primary antibody was used as internal control. The experiment was performed in triplicate.

2.9. Cell viability assay

To detect the potential effect of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin on cell viability, cell counting kit-8 (CCK-8) assay (Beyotime Biotechnology, China) was performed at 48 h post-transfection [49]. Briefly, EPC cells were seeded in 96-well plates for 18 h. Following cell transfection, 10 μl of CCK-8 solution was added to each well. The optical density was measured at 450 nm. The experiment was performed in triplicate.

2.10. Measurement of reactive oxygen species (ROS)

For detection of intracellular ROS production, DCFH-DA probe (Beyotime Biotechnology, China) was used [50]. In brief, EPC cells were seeded in 24-well plates overnight and transfected with above plasmids as above described. Then, cells were stained with DCFH-DA (10 μM) for 20 min in dark and then washed three times with PBS. ROS signalings were detected at 48 h post-transfection by fluorescent microscopy. The experiment was performed in triplicate.

2.11. Mitochondrial membrane potential (MMP) detection assay

For detection of mitochondrial integrity, cell transfection was performed as above. Then, EPC cells were detected by using a mitochondrial membrane potential (MMP) assay kit with JC-1 (Beyotime Biotechnology, China). After washing with JC-1 buffer, fluorescence signalings were detected at 48 h post-transfection by using a fluorescence microscope [51]. The experiment was performed in triplicate.

2.12. Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed in fish cells as previously described [52]. In brief, EPC cells were seeded in 24-well plates for 18 h and co-transfected with PRL-TK, nuclear factor-κB (NF-κB) Luc, activator protein-1 (AP-1) Luc and signal transducers and activators of transcription 3 (STAT3) Luc, respectively. Following 24 h, luciferase reporter activity was performed by using a dual-luciferase reporter assay system (Promega, USA). Relative folds of luciferase activity were normalized to the amount of Renilla luciferase. The results were repeated in triplicate.

2.13. In vivo effect of RCC/WCC/WR-NK-lysin overexpression on gene expressions

Based on previous studies, gene overexpression in fish was performed [22,53,54]. In brief, the above endotoxin-free plasmids were diluted in PBS to 200 ng/μl, respectively. Fish were randomly divided and injected intramuscularly with 100 μl of above endotoxin-free plasmids. Then, spleen were isolated from fish at 5 d post-plasmid administration. Then, isolated total RNA was used for RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin expression by qRT-PCR detection [55]. To determine the expression of immune-related genes, total RNA was prepared from spleen of RCC/WCC/WR-NK-lysin, pcDNA3.1 or PBS-injected fish at 5 d post-plasmid administration as above. Following cDNA synthesis, expressions of NK-lysin, Interleukin-1β-1 (IL-1β-1), Interleukin-1β-2 (IL-1β-2), tumor necrosis factor-α-1 (TNFα-1) and tumor necrosis factor-α-2 (TNFα-2) were detected by qRT-PCR assay. Primers used in this study were shown in Table 1. Primer specificity was confirmed and each sample was analyzed in triplicate to certify the repetitiveness and credibility of experimental results. The experiment was performed in triplicate.

2.14. In vivo effect of RCC/WCC/WR-NK-lysin overexpression on A. Hydrophila infection

To investigate the effect of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin on the growth of *A. hydrophila*, the *in vivo* injection was performed as described above. Following 5 d post-plasmid administration, 6 fish in each group were infected with *A. hydrophila* as described above. Spleen were isolated at 24 h post-infection, then genomic DNA of tissues, including bacterial DNA, was extracted by using a DNA extraction kit (Omega, USA), and the concentration was adjusted to 100 ng/μl qPCR assay was used to detect hlyA gene of *A. hydrophila*, while GAPDH gene was analyzed as the reference gene [56,57]. Bacterial load assays were performed as previously described [58,59]. In brief, isolated tissues (approximately 0.15 g fresh tissue weight) were homogenized in sterile PBS. Then, the homogenates were incubated onto LB agar plates for 16 h, then bacterial colonies were counted. The experiments were performed in triplicate.

2.15. Statistical analyses

The data analysis was measured by using SPSS 18 analysis program and represented as means ± standard deviation. All of the experimental data analyses were subjected to Student's *t*-test or one-way ANOVA (one-way analysis of variance). Further analysis of Duncan's multiple range test, only if the level of P-value < 0.05, the differences were considered statistically significant.

3. Results

3.1. Characterization of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin cDNA sequences

In this study, ORF sequences of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin were 369 bp, encoding 122 amino acid residues with a predicted molecular weight of 13.76 KDa, 13.74 KDa and 13.75 KDa, respectively. As shown in Supplementary Fig. 1A, multiple alignment analysis revealed that domain structures of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin exhibited a close similarity to the counterparts in other vertebrates, comprising two functional domains: a signal peptide (1–17 amino acids) and saposin B domain (47–120 amino acids). In Supplementary Fig. 2B, phylogenetic analysis suggested that RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin sequences were similar to those of other teleost species, suggesting that the evolutionary relationship were in agreement with the concept of the traditional taxonomy. In Supplementary Fig. 1C-E, secondary structure analysis revealed that both RCC-NK-lysin and WCC-NK-lysin contained 4 helices, 5 helix-helix interaction motifs, 3 β-turns, 2 γ-turns and 3 disulfide bonds, while WR-NK-lysin contained 4 helices, 5 helix-helix interaction motifs, 3 β-turns, 3 γ-turns and 3 disulfide bonds. In Supplementary Fig. 1F-H, tertiary structures of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin were 22% identical to d1nkla template with 99.8% confidence and the image was colored by rainbow from N- to *C*-terminus.

3.2. Gene expression profiles of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA

In Fig. 1, tissue-specific RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA expression were detected in all isolated samples (L: liver; I: intestine; TK: trunk kidney; G: gill; M: muscle; B: brain; S: spleen). High-level mRNA expressions of RCC-NK-lysin and WCC-NK-lysin were observed in gill, while the highest expression level of WR-NK-lysin mRNA was observed in spleen. In contrast, the lowest expression levels of RCC-NK-lysin and WCC-NK-lysin mRNA were observed in muscle, while the lowest expression level of WR-NK-lysin mRNA was observed in brain.

Furthermore, expression patterns of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA in liver, trunk kidney and spleen were investigated at 0, 6, 12, 24, 36 and 48 h after *A. hydrophila* challenge. In Fig. 2A-C, RCC-NK-lysin mRNA expressions in liver, trunk kidney and spleen peaked at 6 h following *A. hydrophila* challenge with the highest values of 39.82, 21.18-, 30.82-fold greater than those of the control ($P < 0.05$). In Fig. 2D-F, dramatic increase of WCC-NK-lysin mRNA expressions in liver, trunk kidney and spleen were observed after *A. hydrophila* challenge and reached the peaked level at 48 h with the highest values of 16.70-, 37.92- and 14.33-fold greater than those of the control ($P < 0.05$). In Fig. 2G-I, expressions of WR-NK-lysin mRNA in liver and trunk kidney peaked at 24 h after *A. hydrophila* challenge with the highest values of 42.52- and 59.12-fold greater than those of the control ($P < 0.05$), while WR-NK-lysin mRNA in spleen reached a peaked level at 36 h with the highest value of 67.84-fold greater than that of the control ($P < 0.05$).

Expression profiles of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin in RCCFCs, WCCFCs and WRFCs were investigated at 0, 6, 12, 24, 36 and 48 h following LPS stimulation. In Fig. 3A, expression level of RCC-NK-lysin mRNA in RCCFCs peaked at 6 h following 500 ng/ml LPS challenge, while a high level of RCC-NK-lysin mRNA was observed at 12 h following 1000 ng/ml LPS stimulation with the highest value of 26.05-fold greater than that of the control, followed by a sharp decrease from 24 h to 48 h. In Fig. 3B, a significant fluctuation of WCC-NK-lysin mRNA in WCCFCs was observed from 6 h to 48 h following 500 ng/ml LPS stimulation, whereas a 28.24-fold increase of WCC-NK-lysin mRNA was observed at 6 h after 1000 ng/ml LPS challenge, followed by a gradual decrease from 12 h to 48 h. In Fig. 3C, WRFCs receiving 500 ng/ml LPS

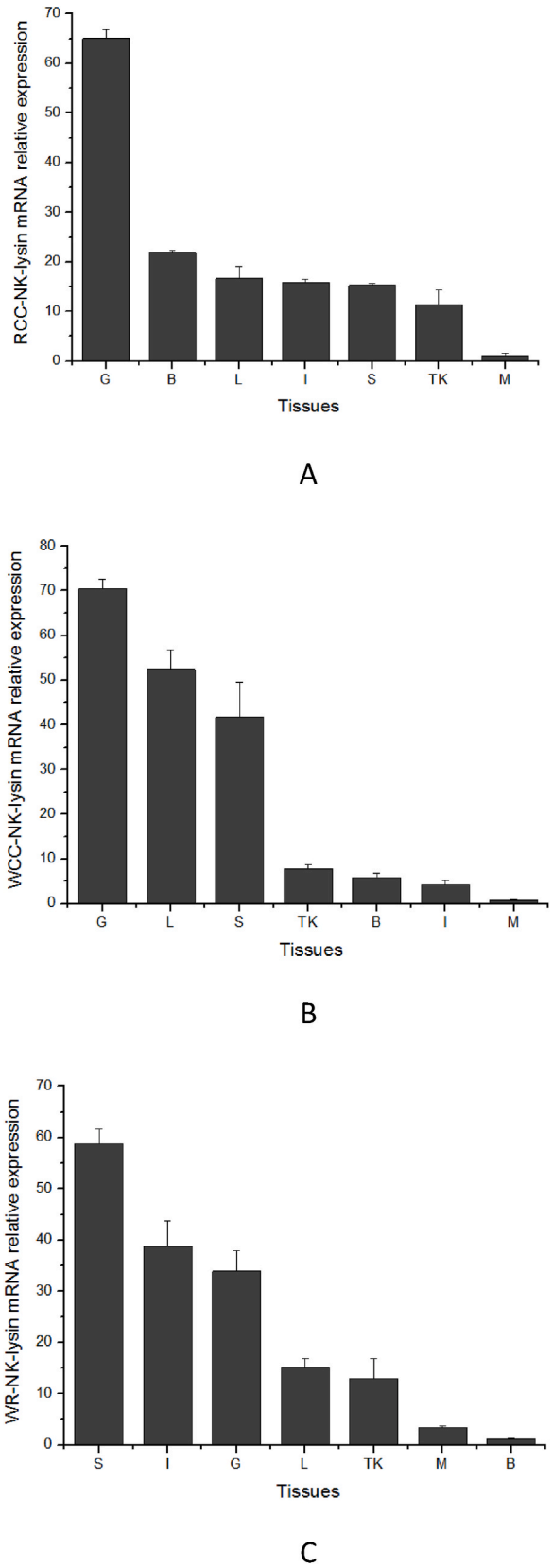

Fig. 1. Tissue-specific expressions of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA. Relative RCC-NK-lysin (A), WCC-NK-lysin (B) and WR-NK-lysin (C) mRNA expression of each tissue was calculated by the $2^{-\triangle\triangle Ct}$ methods using 18 S rRNA as a reference gene, and the relative mRNA level was compared with spleen expression. (L: liver; I: intestine; TK: trunk kidney; G: gill; M: muscle; B: brain; S: spleen).

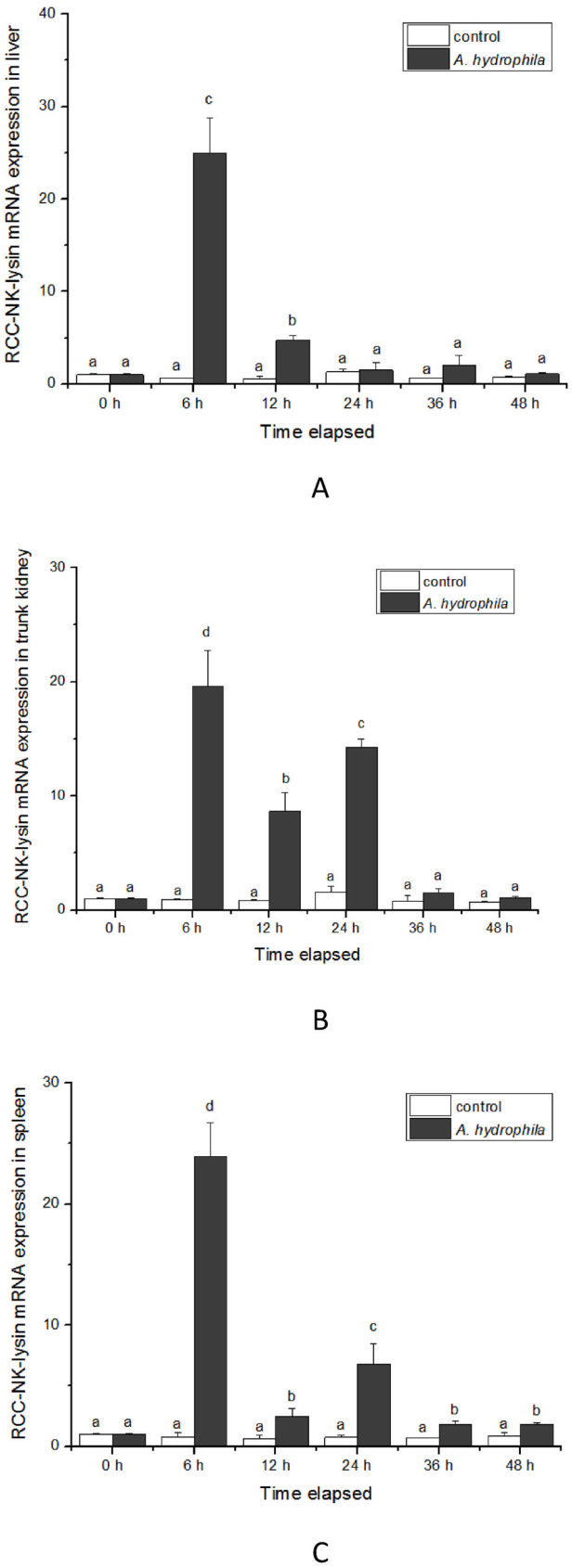

Fig. 2. qRT-PCR analysis of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA expression in liver, trunk kidney and spleen at 0, 6, 12, 24, 36 and 48 h post-challenge. The calculated data (mean ± SD) of six individuals (n = 6) with different letters were significantly different ($P < 0.05$).

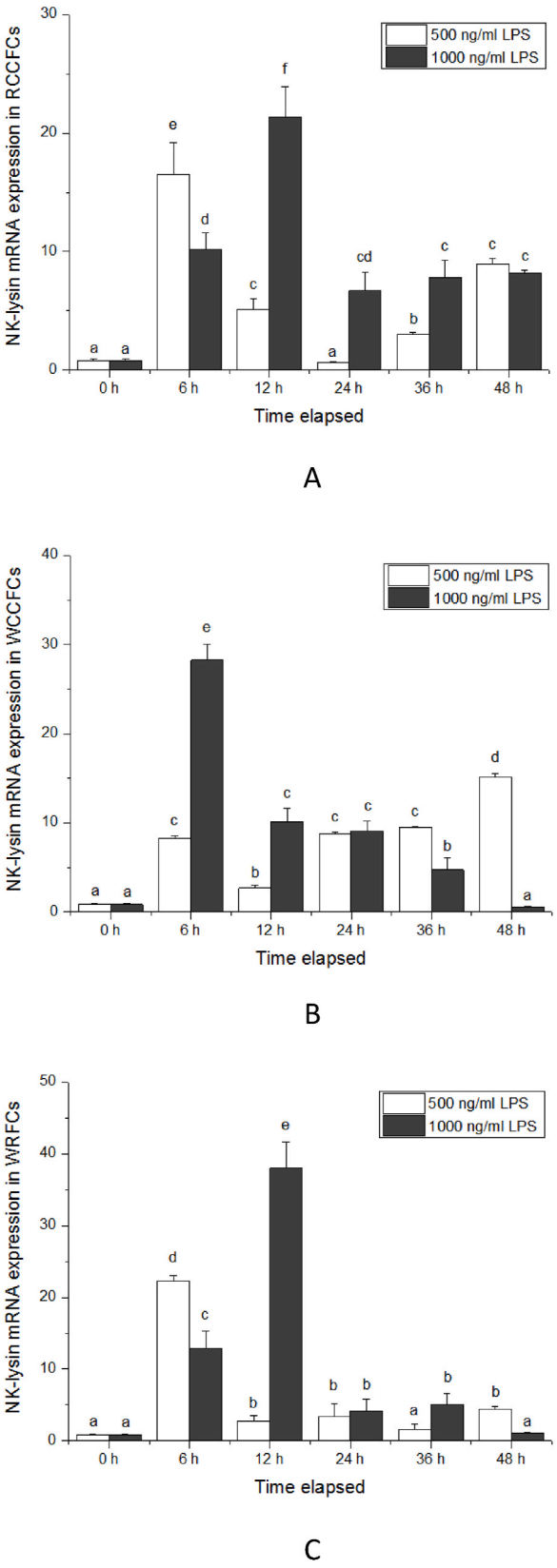

Fig. 3. qRT-PCR analysis of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA expression in RCCFCs, WCCFCs and WRFCs at 0, 6, 12, 24, 36 and 48 h following various doses of LPS stimulation. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

stimulation exhibited a sharp increase of WR-NK-lysin mRNA expression at 6 h, while elevated level of WR-NK-lysin mRNA expression was observed at 12 h following 1000 ng/ml LPS challenge with the peaked level of 37.98-fold greater than that of the control ($P < 0.05$).

3.3. RCC/WCC/WR-NK-lysin lowered cell viability, induced ROS generation and mediated mitochondrial damage in fish cells

Evidences are emerging that pig NK-lysin is a novel effector of CTL and NK cells, which cannot only shows antibacterial activity against *Escherichia coli* and *Bacillus megaterium,* but also exhibits a marked lytic activity against mouse tumor cell lines YAC-1, 3B6 lymphocytes, 1G8 cells and U937 cells under similar half-maximal concentrations [13,18]. In addition, synthesized N29 N peptide from chicken NK-lysin shows a greater antibacterial activity and higher cytotoxic activity of tumor cells with a high helical profile [60]. To investigate the cellular regulation, RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin were overexpressed in fish cells. In Fig. 4A, Recombinant NK-lysin proteins were strongly expressed in RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin expression cells at 48 h post-transfection by comparing with that of the control, respectively. As shown in Fig. 4B-C, a higher level of ROS intensity was observed in RCC/WCC/WR-NK-lysin expression cells at 48 h post-transfection by comparing with that of the control. In Fig. 4D-E, JC-1 dye was predominantly aggregated in the control with a low ratio of green/red intensity, while a strong green fluorescence signaling was observed in RCC/WCC/WR-NK-lysin expression cells at 48 h post-transfection, respectively. To detect the effect of RCC/WCC/WR-NK-lysin on fish cell viability, CCK-8 assays were performed. As shown in Supplementary Fig. 2, cell viability in RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin expression cells decreased to 78.23%, 76.86% and 75.41% at 48 h post-transfection by comparing with the control.

3.4. Effect of RCC/WCC/WR-NK-lysin overexpression on NF-κB, AP-1 and STAT3 promoter activities in fish cells

To detect the effect of RCC/WCC/WR-NK-lysin overexpression on NF-κB, AP-1 and STAT3 promoter activities, luciferase reporter assays were performed. In Fig. 5A-C, overexpression of RCC-NK-lysin could dramatically antagonize AP-1, NF-κB, STAT3 reporter activity in RCCFCs, respectively. 400 ng RCC-NK-lysin plasmids could decrease AP-1, NF-κB, STAT3 activity to 77.62%, 61.60%, 68.90%, while overexpression of 800 ng RCC-NK-lysin plasmids could inhibit their activities to 56.94%, 25.54% and 42.76%, respectively.

In Supplementary Figs. 3A–C, WCC-NK-lysin overexpression could significantly abrogate AP-1, NF-κB, STAT3 reporter activity in WCCFCs,

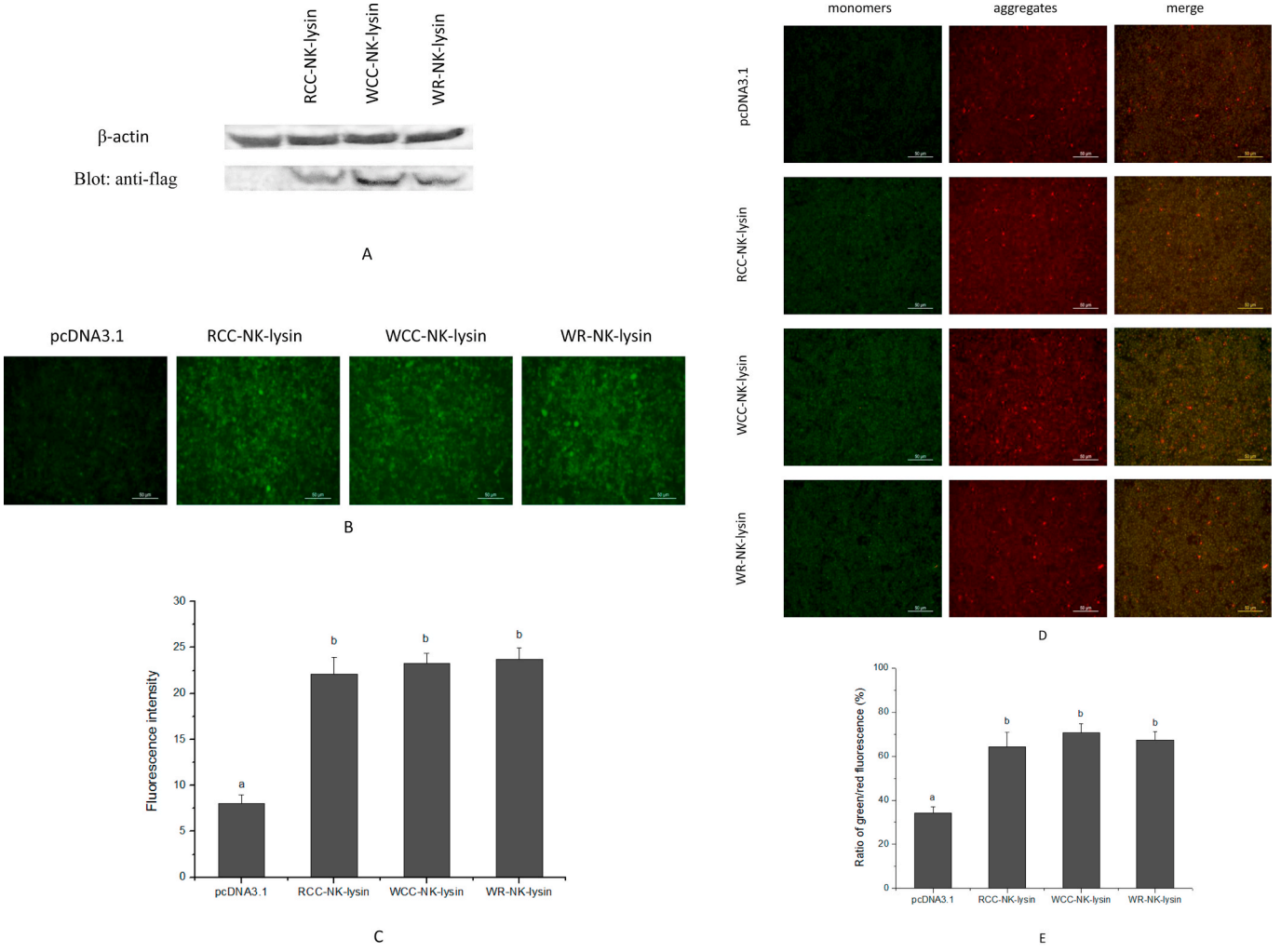

Fig. 4. Overexpression of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin can induce cytotoxic effect in fish cells. (A) Western blotting. Isolated proteins of fish cells transfected with pcDNA3.1 or pcDNA3.1-RCC-NK-lysin, pcDNA3.1-WCC-NK-lysin, pcDNA3.1-WR-NK-lysin plasmids. The anti-β-actin was used as the internal control. (B–C) Detection of ROS generation. Cells were transfected as above described. Following 48 h transfection, the change of ROS generation was performed by using DCFH-DA probe. (D–E) Detection of MMP activity. Cells were transfected as above described. Following 48 h transfection, the change of MMP activity was performed by using a MMP assay kit.

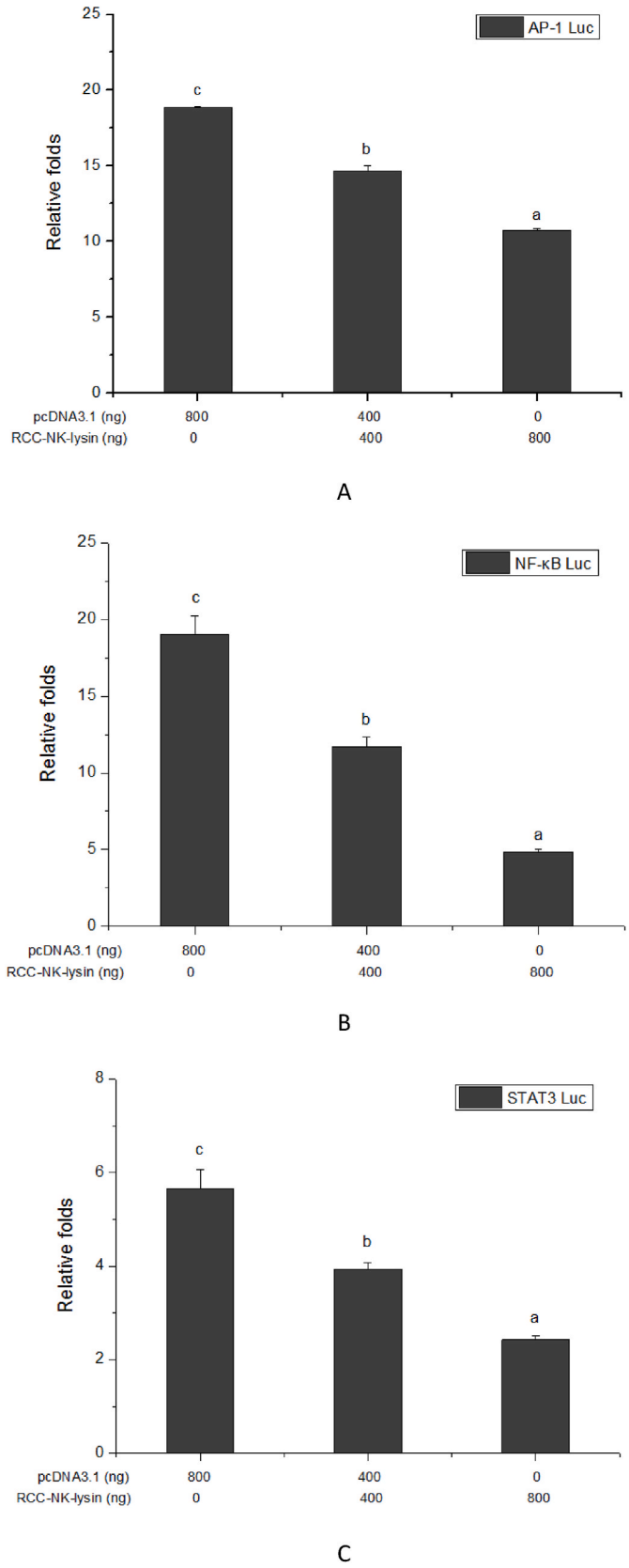

Fig. 5. Effects of RCC-NK-lysin overexpression on the promoter activities. EPCs were co-transfected with pRL-TK, NF-κB-Luc/AP-1-Luc/STAT3-Luc, together with pcDNA3.1 or pcDNA3.1-RCC-NK-lysin. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

respectively. 400 ng WCC-NK-lysin plasmids could attenuate AP-1, NF-κB, STAT3 activity to 68.96%, 57.94% and 63.86%, while 800 ng WCC-NK-lysin plasmids could alleviate their activities to 46.78%, 28.72% and 35.20%, respectively.

In Supplementary Figs. 3D–F, WR-NK-lysin overexpression could sharply reduce AP-1, NF-κB, STAT3 reporter activity in WRFCs, respectively. 400 ng WR-NK-lysin plasmids could decrease AP-1, NF-κB, STAT3 activity to 72.29%, 58.40% and 66.33%, while overexpression of 800 ng WR-NK-lysin plasmids could inhibit their activities to 48.08%, 30.31% and 31.64%, respectively.

3.5. Effect of RCC/WCC/WR-NK-lysin overexpression on immune-related gene expressions in fish

To investigate the biological activity of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin *in vivo*, fish were administrated with RCC-NK-lysin, WCC-NK-lysin or WR-NK-lysin plasmid, while the control vector pcDNA3.1 or PBS was used as the control. As shown in Fig. 6A-C, RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin plasmid injected fish exhibited up-regulated expressions of NK-lysin, IL-1β-1, IL-1β-2, TNFα-1 and TNFα-2 in spleen by comparing with those of the control, respectively.

3.6. Inhibitory effect of RCC/WCC/WR-NK-lysin on the growth of A. Hydrophila in vivo

To investigate the effect of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin on the growth of *A. hydrophila in vivo*, bacterial load and expression of *A. hydrophila* hlyA in spleen were determined at 24 h post-infection. In Fig. 7A and Supplementary Fig. 4A-B, the administration of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin plasmid could sharply reduce bacterial load in spleen at 24 h post-challenge by comparing with those of the control. As shown in Fig. 7B and Supplementary Fig. 4C-D, the relative expression of *A. hydrophila* hlyA in spleen in RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin plasmid administrated group were significantly lower than those of the control, respectively.

4. Discussion

In this study, sequences of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin were cloned from the liver of RCC, WCC and WR, respectively. The deduced RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin amino acid sequences consisted of 122 amino acid residues, including a signal peptide and saposin B domain, suggesting that RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin belonged to saposin-like protein (SAPLIP) family. Sequence alignments revealed that RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin contained highly conserved cysteine residues, which may play an essential role in biological function in fish [22]. Moreover, RCC-NK-lysin and WCC-NK-lysin were detected in isolated samples with high-level expression levels in gill, while the highest level of WR-NK-Lysin expression was observed in spleen, similar to those of Nile tilapia [23] and croaker [61], indicating that RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA were expressed in a broad range of tissues.

Liver, trunk kidney and spleen are playing vital roles in teleostean immunity [62,63]. In this study, we investigated mRNA expression patterns of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin in immune-related tissues following *A. hydrophila* challenge. The elevated levels of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA were detected in liver, trunk kidney and spleen following *A. hydrophila* challenge. In addition, up-regulated expression profiles of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin mRNA were observed in RCCFCs, WCCFCs and WRFCs following various doses of LPS stimulation, which may respond to bacterial infection and LPS stimulation. However, comparative mechanisms linking NK-lysin to immune regulation in fish derived from hybrid fish and its parents are still unclear.

As is well known, AMPs are abundant molecules that can be

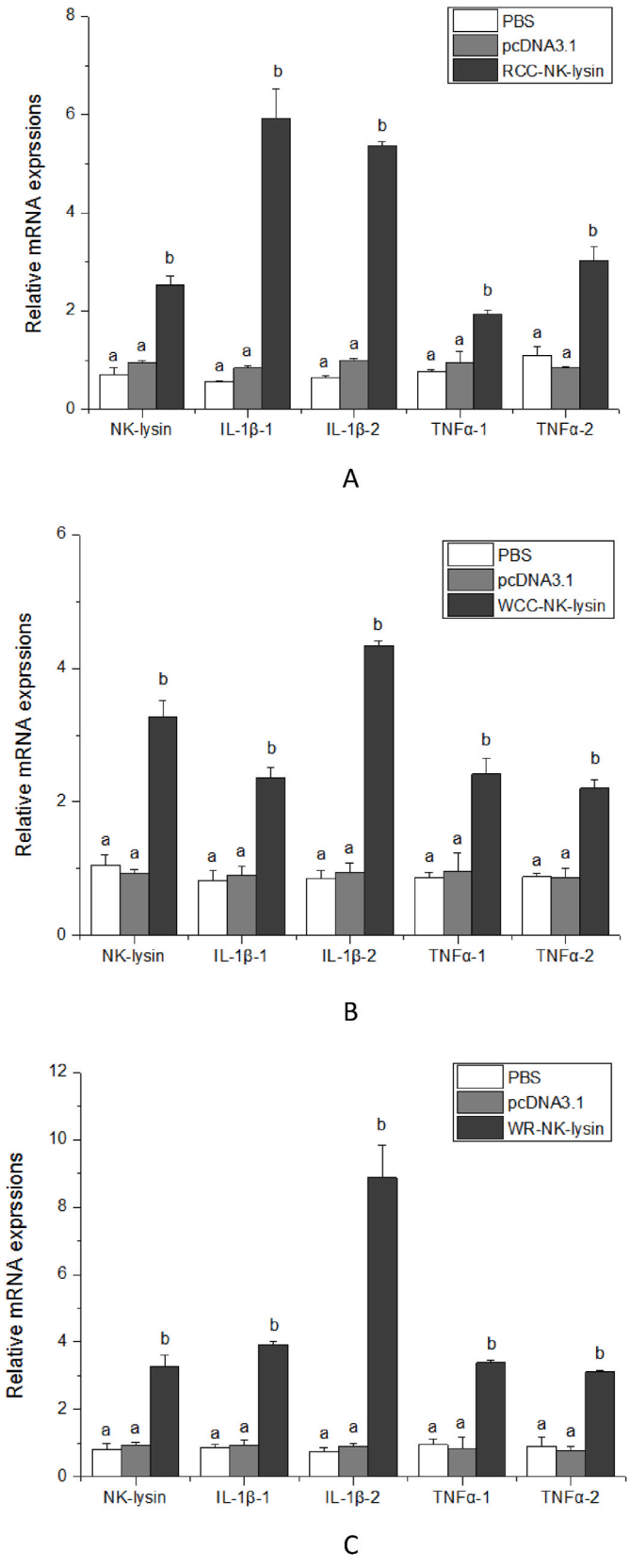

Fig. 6. Relative transcriptional level of immune-related gene in fish at 5 days post DNA injection. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

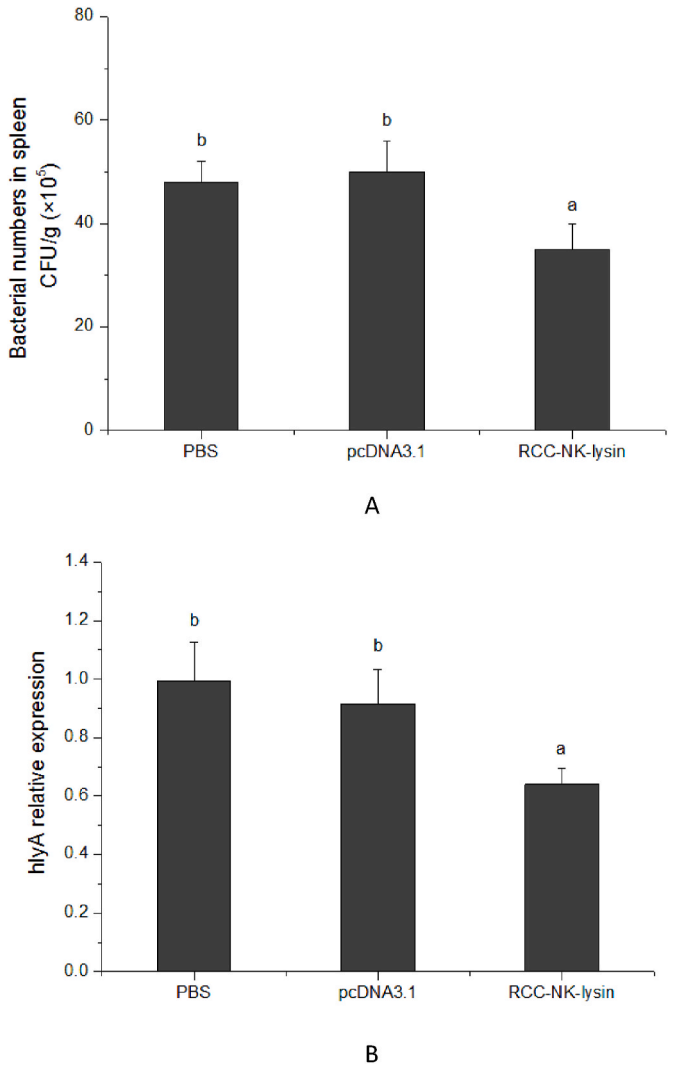

Fig. 7. Effect of RCC-NK-lysin overexpression on bacterial infection. (A) Bacterial load assay. Fish were administrated with PBS, pcDNA3.1 or pcDNA3.1-RCC-NK-lysin and then infected with *A. hydrophila*. Bacterial numbers in spleen were determined at 24 h post-infection. The bacterial colonies were counted. (B) Detection of *A. hydrophila* hlyA gene relative expression by qRT-PCR. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

produced by many tissues and cell types in a variety of plants and animals [64]. In general, amino acid composition, amphipathicity, cationic charge and molecular weight of AMPs can facilitate their insertion into membrane bilayers and form pores, which may abrogate the synthesis of nucleic acids, proteins and cell walls [7]. Evidences are emerging that both granulysin and NK-lysin can elicit antibacterial activity and exhibit cytotoxic effect on eukaryotic cells with a substantial sequence similarity in spite of their enormous evolutionary distance [65]. Previous studies synthesized croaker NK-lysin peptide can exhibit a strong antibacterial activity *in vitro* [61]. In addition, the administration of On-NK-lysin plasmid can improve immune defense against *S. agalactiae* infection [23]. However, comparative mechanisms linking NK-lysin to immune regulation in fish derived from hybrid offspring and its parents are still unclear. In this study, fish administrated with RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin plasmid showed a higher level of NK-lysin expressions compared with those of the control. In addition, The up-regulated expressions of IL-1β-1, IL-1β-2, TNFα-1 and TNFα-2 in spleen were observed in RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin plasmid administration groups, respectively. IL-1β is a pro-inflammatory

cytokine that is involved in regulation of chronic disease processes [66] and organic anion transporters in human hepatic cells [67], while TNF can regulate systemic defense against invading bacteria via modulation of immune cells [68–70]. Besides, bacterial loads in spleen in RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin plasmid group were significantly lower than those of the control. These studies suggested that RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin could exhibit an antibacterial activity and regulate immune response.

Among known SAPLIP family, NK-lysin is considered as cationic antibacterial peptide produced by cytotoxic lymphocytes with a high homology to granulysin [71]. Previous studies indicate that granulysin can directly disrupt membrane potential in mitochondria, trigger release of cytochrome *c* and activating caspase activation [72,73]. In this study, overexpression of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin in fish cells could significantly lower the cell viability, trigger ROS generation and regulate the MMP activity. Moreover, luciferase report assay revealed that overexpression of RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin could reduce NF-κB, AP-1 and STAT3 promoter activity in fish cells.

NF-κB plays a key role in the determination of the choice between cell survival or cell death [74]. In general, NF-κB activation by *in vitro* stimuli such as TNF, ionizing radiation or daunorubicin can confer protection against cell death by suppressing apoptosis [75]. Previous studies indicate that overexpression of NF-κB subunit can antagonize p53-induced Bax promoter activity in HCT116 cells, while its blockade can increase apoptotic killing [76,77]. Additionally, NF-κB is one of a key substrates that can be cleaved by induced caspase activation, then suppressing the protective effect of NF-κB and abrogating cell survival signalings [78,79].

AP-1 complexes comprise dimmers of Fos, Jun and ATF subunits may participate in cellular proliferation, transformation and death [80,81]. In general, AP-1 may play a multiple role in distinct apoptotic pathways in many cell types [82]. Recent studies indicate that cobalt chloride-induced apoptosis is accompanied with ROS-induced AP-1 activity in PC12 cells [83]. In addition, the administration of triptolide, a NF-κB inhibitor, can decrease cell proliferative activity, induce apoptosis and suppress NF-κB and AP-1 transactivation in AGS cells [84].

STATs serving as multifaceted oncogene can mediate cytokine- and growth factor-directed transcription regulation and active STAT3 signaling may play an important role in cellular transformation and apoptotic process [85]. STAT3 activation can render cells more resistant to apoptosis in human myeloma cells [86], whereas blockade of STAT3 activity can dramatically antagonize anti-apoptotic effect of IL-6 in human cancer cells [87]. In addition, STAT3 activation may also play a regulatory role in expression levels of apoptosis-related genes, then modulating the oncogenic transformation via cell survival signaling [88].

Thus, NF-κB, AP-1 and STAT3 signalings are indispensable for the cellular survival. These studies demonstrated that RCC-NK-lysin, WCC-NK-lysin and WR-NK-lysin sharing the conserved domains could exert their cytotoxic effect on fish cells by disrupting survival signalings.

In summary, we compared architectures of RCC/WCC/WR-NK-lysin. Then, we identified up-regulated expressions of RCC/WCC/WR-NK-lysin mRNA in tissues after bacterial infection or in fish cells following various doses of LPS stimulation. Overexpression of RCC/WCC/WR-NK-lysin could lower cell viability, induce ROS generation, trigger the loss of MMP activity and attenuate NF-κB, AP-1 and STAT3 promoter activity in fish cells, respectively. Moreover, the administration of RCC-NK-lysin, WCC-lysin and WR-NK-lysin plasmid stimulated immune-related gene expressions and conferred protection against *A. hydrophila* infection. Our results indicated that induction of cytotoxic and antibacterial activity by NK-lysin were similar in hybrid offspring and its parents.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.06.015.

References

[1] Z. Qi, Y. Zhang, Z.-F. Chen, C. Yang, Y. Song, X. Liao, et al., Chemical identity and cardiovascular toxicity of hydrophobic organic components in PM2. 5, Ecotoxicol. Environ. Saf. 201 (2020) 110827.
[2] Y.Y. Sung, T.H. MacRae, P. Sorgeloos, B. P, Stress response for disease control in aquaculture, Rev. Aquacult. 3 (2011) 120–137.
[3] B. Magnadottir, Immunological control of fish diseases, Mar. Biotechnol. 12 (2010) 361–379.
[4] H.J. Muller-Eberhard, The membrane attack complex of complement, Annu. Rev. Immunol. 4 (1986) 503–528.
[5] M. Wenzel, A.I. Chiriac, A. Otto, D. Zweytick, C. May, C. Schumacher, et al., Small cationic antimicrobial peptides delocalize peripheral membrane proteins, Proc. Natl. Acad. Sci. Unit. States Am. 111 (2014) E1409–E1418.
[6] M.L. Mangoni, J.M. Saugar, M. Dellisanti, D. Barra, M. Simmaco, L. Rivas, Temporins, small antimicrobial peptides with leishmanicidal activity, J. Biol. Chem. 280 (2005) 984–990.
[7] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238.
[8] R.E. Hancock, H.-G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551.
[9] R.E. Hancock, Cationic peptides: effectors in innate immunity and novel antimicrobials, Lancet Infect. Dis. 1 (2001) 156–164.
[10] J. Ageitos, A. Sánchez-Pérez, P. Calo-Mata, T. Villa, Antimicrobial peptides (AMPs): ancient compounds that represent novel weapons in the fight against bacteria, Biochem. Pharmacol. 133 (2017) 117–138.
[11] Y. Lai, A.E. Villaruz, M. Li, D.J. Cha, D.E. Sturdevant, M. Otto, The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci, Mol. Microbiol. 63 (2007) 497–506.
[12] R. Rathinakumar, W.F. Walkenhorst, W.C. Wimley, Broad-spectrum antimicrobial peptides by rational combinatorial design and high-throughput screening: the importance of interfacial activity, J. Am. Chem. Soc. 131 (2009) 7609–7617.
[13] M. Andersson, H. Gunne, B. Agerberth, A. Boman, T. Bergman, R. Sillard, et al., NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity, EMBO J. 14 (1995) 1615–1625.
[14] E. Liepinsh, M. Andersson, J.-M. Ruysschaert, G. Otting, Saposin fold revealed by the NMR structure of NK-lysin, Nat. Struct. Biol. 4 (1997) 793–795.
[15] A.R. French, W.M. Yokoyama, Natural killer cells and viral infections, Curr. Opin. Immunol. 15 (2003) 45–51.
[16] J. Li, Y. Han, K. Jin, Y. Wan, S. Wang, B. Liu, et al., Dynamic changes of cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and natural killer T (NKT) cells in patients with acute hepatitis B infection, Virol. J. 8 (2011) 1–8.
[17] M. Andersson, T. Curstedt, H. Jörnvall, J. Johansson, An amphipathic helical motif common to tumourolytic polypeptide NK-lysin and pulmonary surfactant polypeptide SP-B, FEBS Lett. 362 (1995) 328–332.
[18] M. Andersson, A. Holmgren, G. Spyrou, NK-lysin, a disulfide-containing effector peptide of T-lymphocytes, is reduced and inactivated by human thioredoxin reductase: implication for a protective mechanism against NK-lysin cytotoxicity, J. Biol. Chem. 271 (1996) 10116–10120.
[19] N. dos Santos, M. Silva, Ad Vale, Fish and apoptosis: studies in disease and pharmaceutical design, Curr. Pharmaceut. Des. 14 (2008) 170–183.
[20] S.W. Luo, W. Wei, Molecular characterization of complement 9 in Epinephelus coioides and differential expression analysis of classical complement genes following Vibrio alginolyticus challenge, Ecotoxicology 29 (2020) 837–845.
[21] Q. Wang, B. Bao, Y. Wang, E. Peatman, Z. Liu, Characterization of a NK-lysin antimicrobial peptide gene from channel catfish, Fish Shellfish Immunol. 20 (2006) 419–426.
[22] M. Zhang, H. Long, L. Sun, A NK-lysin from Cynoglossus semilaevis enhances antimicrobial defense against bacterial and viral pathogens, Dev. Comp. Immunol. 40 (2013) 258–265.
[23] Y. Huang, Q. Zheng, J. Niu, J. Tang, B. Wang, E.D. Abarike, et al., NK-lysin from Oreochromis niloticus improves antimicrobial defence against bacterial pathogens, Fish Shellfish Immunol. 72 (2018) 259–265.

[24] F.-F. Ding, C.-H. Li, J. Chen, Molecular characterization of the NK-lysin in a teleost fish, Boleophthalmus pectinirostris: antimicrobial activity and immunomodulatory activity on monocytes/macrophages, Fish Shellfish Immunol. 92 (2019) 256–264.
[25] R.L. Rogers, T. Bedford, D.L. Hartl, Formation and longevity of chimeric and duplicate genes in Drosophila melanogaster, Genetics 181 (2009) 313–322.
[26] S. Liu, J. Luo, J. Chai, L. Ren, Y. Zhou, F. Huang, et al., Genomic incompatibilities in the diploid and tetraploid offspring of the goldfish× common carp cross, Proc. Natl. Acad. Sci. Unit. States Am. 113 (2016) 1327–1332.
[27] H. Koyama, T. Ito, T. Nakanishi, K. Sekimizu, Stimulation of RNA polymerase II transcript cleavage activity contributes to maintain transcriptional fidelity in yeast, Gene Cell. 12 (2007) 547–559.
[28] A. Šimková, L. Vojtek, K. Halačka, P. Hyršl, L. Vetešník, The effect of hybridization on fish physiology, immunity and blood biochemistry: a case study in hybridizing Cyprinus carpio and Carassius gibelio (Cyprinidae), Aquaculture 435 (2015) 381–389.
[29] A. Šimková, M. Dávidová, I. Papoušek, L. Vetešník, Does interspecies hybridization affect the host specificity of parasites in cyprinid fish? Parasites Vectors 6 (2013) 95.
[30] Z. Li, Z.W. Wang, Y. Wang, J.F. Gui, Crucian Carp and Gibel Carp Culture. *Aquaculture In China: Success Stories and Modern Trends*, 2018, pp. 149–157.
[31] J. Luo, Y. Gao, W. Ma, X. Bi, S. Wang, J. Wang, et al., Tempo and mode of recurrent polyploidization in the Carassius auratus species complex (Cypriniformes, Cyprinidae), Heredity 112 (2014) 415–427.
[32] Q. Liu, J. Liu, Q. Liang, Y. Qi, M. Tao, C. Zhang, et al., A hybrid lineage derived from hybridization of Carassius cuvieri and Carassius auratus red var. and a new type of improved fish obtained by back-crossing, Aquaculture 505 (2019) 173–182.
[33] D. Marcogliese, The impact of climate change on the parasites and infectious diseases of aquatic animals, Rev Sci Tech 27 (2008) 467–484.
[34] Q. Liu, Y. Qi, Q. Liang, X. Xu, F. Hu, J. Wang, et al., The chimeric genes in the hybrid lineage of Carassius auratus cuvieri (♀)× Carassius auratus red var.(♂), Sci. China Life Sci. 61 (2018) 1079–1089.
[35] S.-W. Luo, H. Kang, J.-R. Kong, R.-C. Xie, Y. Liu, W.-N. Wang, et al., Molecular cloning, characterization and expression analysis of (B-cell lymphoma-2) Bcl-2 in the orange-spotted grouper (Epinephelus coioides), Dev. Comp. Immunol. 76 (2017) 150–162.
[36] S.-W. Luo, H. Kang, R.-C. Xie, W. Wei, Q-j Liang, Y. Liu, et al., N-terminal domain of EcC1INH in Epinephelus coioides can antagonize the LPS-stimulated inflammatory response, Fish Shellfish Immunol. 84 (2019) 8–19.
[37] S.-W. Luo, K.-K. Luo, S.-J. Liu, ITLN in diploid hybrid fish (Carassius auratus cuvieri♀× Carassius auratus red var♂) is involved in host defense against bacterial infection, Dev. Comp. Immunol. 103 (2020) 103520.
[38] S.-W. Luo, Z.-W. Mao, Z.-Y. Luo, N.-X. Xiong, K.-K. Luo, S.-J. Liu, et al., Chimeric ferritin H in hybrid crucian carp exhibit a similar down-regulation in lipopolysaccharide-induced NF-κB inflammatory signal in comparison with Carassius cuvieri and Carassius auratus red var, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 241 (2021) 108966.
[39] Y. Jiang, L. Liu, S. Yang, Y. Cao, X. Song, J. Xiao, et al., Black carp PRMT6 inhibits TBK1-IRF3/7 signaling during the antiviral innate immune activation, Fish Shellfish Immunol. 93 (2019) 108–115.
[40] C. Fierro-Castro, L. Barrioluengo, P. López-Fierro, B. Razquin, A. Villena, Fish cell cultures as in vitro models of inflammatory responses elicited by immunostimulants. Expression of regulatory genes of the innate immune response, Fish Shellfish Immunol. 35 (2013) 979–987.
[41] S.-W. Luo, N.-X. Xiong, Z.-Y. Luo, K.-K. Luo, S.-J. Liu, C. Wu, et al., Effect of Lipopolysaccharide (LPS) stimulation on apoptotic process and oxidative stress in fibroblast cell of hybrid crucian carp compared with those of Carassius cuvieri and Carassius auratus red var, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 248 (2021) 109085.
[42] S. Luo, F. Xie, Y. Liu, W.-N. Wang, Molecular cloning, characterization and expression analysis of complement component C8 beta in the orange-spotted grouper (Epinephelus coioides) after the Vibrio alginolyticus challenge, Gene 558 (2015) 291–298.
[43] S.-W. Luo, L. Cai, Z.-H. Qi, C. Wang, Y. Liu, W.-N. Wang, Effects of a recombinant complement component C3b functional fragment α 2 MR (α 2-macroglobulin receptor) additive on the immune response of juvenile orange-spotted grouper (Epinephelus coioides) after the exposure to cold shock challenge, Fish Shellfish Immunol. 45 (2015) 346–356.
[44] S.-W. Luo, W.-N. Wang, L. Cai, Z.-H. Qi, C. Wang, Y. Liu, et al., Effects of aDissostichus mawsoni-CaM recombinant proteins feed additive on the juvenile orange-spotted grouper (Epinephelus coioides) under the acute low temperature challenge, Fish Physiol. Biochem. 41 (2015) 1345–1358.
[45] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method, Methods 25 (2001) 402–408.
[46] S. Luo, Y. Huang, F. Xie, X. Huang, Y. Liu, W. Wang, et al., Molecular cloning, characterization and expression analysis of PPAR gamma in the orange-spotted grouper (Epinephelus coioides) after the Vibrio alginolyticus challenge, Fish Shellfish Immunol. 43 (2015) 310–324.
[47] S.-W. Luo, W. Wei, P. Yang, C.-M. Lai, Q-j Liang, Y. Liu, et al., Characterization of a CD59 in orange-spotted grouper (Epinephelus coioides), Fish Shellfish Immunol. 89 (2019) 486–497.
[48] G.-H. Cha, S.-W. Luo, Z-h Qi, Y. Liu, W.-N. Wang, Optimal Conditions for Expressing a Complement Component 3b Functional Fragment (α2-Macroglobulin Receptor) Gene from Epinephelus coioides in Pichia pastoris. *Protein Expression and Purification*, 2015.
[49] S.-W. Luo, W.-N. Wang, R.-C. Xie, F.-X. Xie, J.-R. Kong, Y.-C. Xiao, et al., Molecular cloning and characterization of PTEN in the orange-spotted grouper (Epinephelus coioides), Fish Shellfish Immunol. 58 (2016) 686–700.
[50] X. Huang, Y. Huang, Z. Ouyang, L. Xu, Y. Yan, H. Cui, et al., Singapore grouper iridovirus, a large DNA virus, induces nonapoptotic cell death by a cell type dependent fashion and evokes ERK signaling, Apoptosis 16 (2011) 831–845.
[51] S.-W. Luo, W.-N. Wang, Z.-M. Sun, F.-X. Xie, J.-R. Kong, Y. Liu, et al., Molecular cloning, characterization and expression analysis of (B-cell lymphoma-2 associated X protein) Bax in the orange-spotted grouper (Epinephelus coioides) after the Vibrio alginolyticus challenge, Dev. Comp. Immunol. 60 (2016) 66–79.
[52] R. Gao, Y. Huang, X. Huang, L. Guan, S. Wei, Y. Zhou, et al., Molecular cloning and characterization of two types of IκBα orthologues in orange-spotted grouper, Epinephelus coioides, Fish Shellfish Immunol. 38 (2014) 101–110.
[53] C.-J. Chang, B. Sun, B. Robertsen, Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model, Vaccine 33 (2015) 2442–2448.
[54] M. Guo, X. Tang, X. Sheng, J. Xing, W. Zhan, The immune adjuvant effects of flounder (Paralichthys olivaceus) interleukin-6 on E. tarda subunit vaccine OmpV, Int. J. Mol. Sci. 18 (2017) 1445.
[55] J.W. Pridgeon, P.H. Klesius, Apolipoprotein A1 in channel catfish: transcriptional analysis, antimicrobial activity, and efficacy as plasmid DNA immunostimulant against Aeromonas hydrophila infection, Fish Shellfish Immunol. 35 (2013) 1129–1137.
[56] S.-W. Luo, K.-K. Luo, S.-J. Liu, A novel LEAP-2 in diploid hybrid fish (Carassius auratus cuvieri♀× Carassius auratus red var.♂) confers protection against bacteria-stimulated inflammatory response, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 228 (2020) 108665.
[57] L. Wang, C. Fan, W. Xu, Y. Zhang, Z. Dong, J. Xiang, et al., Characterization and functional analysis of a novel C1q-domain-containing protein in Japanese flounder (Paralichthys olivaceus), Dev. Comp. Immunol. 67 (2017) 322–332.
[58] H.-X. Li, X.-J. Lu, C.-H. Li, J. Chen, Molecular characterization of the liver-expressed antimicrobial peptide 2 (LEAP-2) in a teleost fish, Plecoglossus altivelis: antimicrobial activity and molecular mechanism, Mol. Immunol. 65 (2015) 406–415.
[59] S.-W. Luo, Z.-Y. Luo, T. Yan, K.-K. Luo, P.-H. Feng, S.-J. Liu, Antibacterial and immunoregulatory activity of a novel hepcidin homologue in diploid hybrid fish (Carassius auratus cuvieri♀× Carassius auratus red var♂), Fish Shellfish Immunol. 98 (2020) 551–563.
[60] M.O. Lee, E.-H. Kim, H.-J. Jang, M.N. Park, H.-J. Woo, J.Y. Han, et al., Effects of a single nucleotide polymorphism in the chicken NK-lysin gene on antimicrobial activity and cytotoxicity of cancer cells, Proc. Natl. Acad. Sci. Unit. States Am. 109 (2012) 12087–12092.
[61] Q.-J. Zhou, J. Wang, M. Liu, Y. Qiao, W.-S. Hong, Y.-Q. Su, et al., Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish Larimichthys crocea, Fish Shellfish Immunol. 55 (2016) 195–202.
[62] C.M. Press, Ø. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (1999) 309–318.
[63] L. Chen, Q. Li, J. Su, C. Yang, Y. Li, Y. Rao, Trunk kidney of grass carp (Ctenopharyngodon idella) mediates immune responses against GCRV and viral/bacterial PAMPs in vivo and in vitro, Fish Shellfish Immunol. 34 (2013) 909–919.
[64] T. Ganz, Defensins: antimicrobial peptides of innate immunity, Nat. Rev. Immunol. 3 (2003) 710–720.
[65] H. Bruhn, M. Leippe, Comparative modeling of amoebapores and granulysin based on the NK-lysin structure structural and functional implications, Biol. Chem. 380 (1999) 1001–1007.
[66] A. Smith, L. Keen, M. Billingham, M. Perry, C. Elson, J. Kirwan, et al., Extended haplotypes and linkage disequilibrium in the IL1R1–IL1A–IL1B–IL1RN gene cluster: association with knee osteoarthritis, Gene Immun. 5 (2004) 451–460.
[67] M. Le Vee, P. Gripon, B. Stieger, O. Fardel, Down-regulation of organic anion transporter expression in human hepatocytes exposed to the proinflammatory cytokine interleukin 1β, Drug Metabol. Dispos. 36 (2008) 217–222.
[68] N.V. Serbina, T.P. Salazar-Mather, C.A. Biron, W.A. Kuziel, E.G. Pamer, TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection, Immunity 19 (2003) 59–70.
[69] R. Malaviya, T. Ikeda, E. Ross, S.N. Abraham, Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α, Nature 381 (1996) 77–80.
[70] K.O. Kisich, M. Higgins, G. Diamond, L. Heifets, Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils, Infect. Immun. 70 (2002) 4591–4599.
[71] D. Andreu, C. Carre±O, C. Linde, H.G. Boman, M. Andersson, Identification of an anti-mycobacterial domain in NK-lysin and granulysin, Biochem. J. 344 (1999) 845–849.
[72] A.A. Kaspar, S. Okada, J. Kumar, F.R. Poulain, K.A. Drouvalakis, A. Kelekar, et al., A distinct pathway of cell-mediated apoptosis initiated by granulysin, J. Immunol. 167 (2001) 350–356.
[73] S. Gamen, D.A. Hanson, A. Kaspar, J. Naval, A.M. Krensky, A. Anel, Granulysin-induced apoptosis. I. Involvement of at least two distinct pathways, J. Immunol. 161 (1998) 1758–1764.
[74] M. Karin, A. Lin, NF-κB at the crossroads of life and death, Nat. Immunol. 3 (2002) 221–227.
[75] J. Kucharczak, M.J. Simmons, Y. Fan, C. Gélinas, To be, or not to be: NF-κB is the answer–role of Rel/NF-κB in the regulation of apoptosis, Oncogene 22 (2003) 8961–8982.
[76] M. Bentires-Alj, E. Dejardin, P. Viatour, C. Van Lint, B. Froesch, J.C. Reed, et al., Inhibition of the NF-kB transcription factor increases Bax expression in cancer cell lines, Oncogene 20 (2001) 2805–2813.

[77] C.-Y. Wang, M.W. Mayo, A.S. Baldwin, TNF-and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB, Science 274 (1996) 784–787.
[78] B.W. Johnson, L.H. Boise, Bcl-2 and caspase inhibition cooperate to inhibit tumor necrosis factor-α-induced cell death in a Bcl-2 cleavage-independent fashion, J. Biol. Chem. 274 (1999) 18552–18558.
[79] M. Barkett, J.E. Dooher, L. Lemonnier, L. Simmons, J.N. Scarpati, Y. Wang, et al., Three mutations in v-Rel render it resistant to cleavage by cell-death protease caspase-3, Biochim. Biophys. Acta Gen. Subj. 1526 (2001) 25–36.
[80] M. Karin, Z-g Liu, E. Zandi, AP-1 function and regulation, Curr. Opin. Cell Biol. 9 (1997) 240–246.
[81] M. Ameyar, M. Wisniewska, J. Weitzman, A role for AP-1 in apoptosis: the case for and against, Biochimie 85 (2003) 747–752.
[82] D. Liebermann, B. Gregory, B. Hoffman, AP-1 (Fos/Jun) transcription factors in hematopoietic differentiation and apoptosis, Int. J. Oncol. 12 (1998) 685–1385.
[83] W. Zou, M. Yan, W. Xu, H. Huo, L. Sun, Z. Zheng, et al., Cobalt chloride induces PC12 cells apoptosis through reactive oxygen species and accompanied by AP-1 activation, J. Neurosci. Res. 64 (2001) 646–653.
[84] X.-H. Jiang, B. Wong, M. Lin, G.-H. Zhu, H.-F. Kung, S.-H. Jiang, et al., Functional p53 is required for triptolide-induced apoptosis and AP-1 and nuclear factor-kB activation in gastric cancer cells, Oncogene 20 (2001) 8009–8018.
[85] J.F. Bromberg, M.H. Wrzeszczynska, G. Devgan, Y. Zhao, R.G. Pestell, C. Albanese, et al., Stat3 as an oncogene, Cell 98 (1999) 295–303.
[86] R. Catlett-Falcone, T.H. Landowski, M.M. Oshiro, J. Turkson, A. Levitzki, R. Savino, et al., Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells, Immunity 10 (1999) 105–115.
[87] Y. Liu, P.-K. Li, C. Li, J. Lin, Inhibition of STAT3 signaling blocks the anti-apoptotic activity of IL-6 in human liver cancer cells, J. Biol. Chem. 285 (2010) 27429–27439.
[88] M. Nielsen, C. Kaestel, K. Eriksen, A. Woetmann, T. Stokkedal, K. Kaltoft, et al., Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells, Leukemia (1999), 08876924.