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Chitosan reduces the protective effects of *IFN-γ2* on grass carp (*Ctenopharyngodon idella*) against *Flavobacterium columnare* infection due to excessive inflammation

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ABSTRACT

IFN-γ is an immunomodulatory factor that has been extensively studied in phenotypes of mammalian macrophages and multifarious inflammatory responses. Usually these studies relied on the classical synergistic activation of *IFN-γ* with LPS (LipoPolySaccharides). However, non-mammalian vertebrates, and in particular fish, are not very susceptible to LPS, and easily acquire tolerance upon repeated exposure. Therefore, for studies in fish, it is necessary to replace the classical *IFN-γ* + LPS immune system activation method, and find other pathogen-associated molecular patterns (PAMPs) capable of stimulating the fish immune system. Here we used an important farmed fish species, *Ctenopharyngodon idella*, to study the effects of *CiIFN-γ2* (*C. idella IFN-γ2*) and chitosan (CS) on its immune responses *in vivo* and *vitro*. Our results showed that the combination of CS and *CiIFN-γ2* significantly enhanced the activation of macrophages, with an activation intensity even stronger than in *CiIFN-γ2* and *CiIFN-γ2* + LPS groups. *In vivo*, injection of *CiIFN-γ2* could improve the survival rate of *C. idella* infected with *Flavobacterium columnare*, while a combined injection of *CiIFN-γ2* + CS only improved protection in the early stages after the challenge. Notably, both injections reduced the bacterial load of viscera and improved the levels of several plasma parameters (TP, T-SOD, LA, and NO). However, a dramatic up-regulation of inflammatory factors, severe inflammatory damage in the intestines and hepatopancreas, and increased mortality in late stages of infection were observed in the *CiIFN-γ2* + CS group. Our findings provide new insights into the macrophage activation phenotypes and inflammatory responses in fish. They also demonstrate that *CiIFN-γ2* could be used as a potential immunopotentiator, but not in combination with CS. This suggests that selection of immunological adjuvants should be carefully tested experimentally.

1. Introduction

As an immunomodulatory factor, IFN-γ is capable of activating the classically activated macrophages and promoting immune responses [1,2]. Previous research has shown that the *IFN-γ2* gene in fish is functionally homologous to the mammalian IFN-γ [3,4]. This gene has been cloned and characterized in a variety of fish species [5–8]. Typical for IFN-γ is its high synergistic proinflammatory response after a stimulation with LPS (Lipopolysaccharides) [9,10]. However, non-mammalian vertebrates, and in particular fish, are not very susceptible to the toxic effects of LPS (that lead to septic shock), and in some cases even acquire tolerance to LPS upon repeated exposure [11]. This might be due to the fact that the TLR4 receptor complex, as we know it from mammals, may not be functional in fish [12,13]. Therefore, it is likely that in fish there is another PAMP that is recognized by *IFN-γ2*, which then leads to significantly enhanced macrophage activation [14]. Notably, it was found in previous studies that chitosan (CS), a deacetylate derivative of chitin that can be found in the cell wall of some fungi, has the ability to activate the NOD-like receptor family, which are widespread in fish, and induce an inflammatory response [14–16]. Some evidence shows that chitosan has the ability to promote leukocyte phagocytosis and antibody production in aquatic animals [17,18]. Therefore, we hypothesised that fish are more sensitive to CS than to LPS, and that chitosan might synergize with IFN-γ2 to enhance the

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activation intensity of macrophages and induce an inflammatory response.

Inflammatory macrophages are characterized by mediating host's defense from a variety of bacteria, protozoa and viruses; driven and/or reinforced by a series of pro-inflammatory cytokines, *iNOS*, and *MHCII* [19–21]. However, these macrophages produce reactive oxygen and inflammatory factors, including NO, *IL-1β*, *TNF-α*, *IL-6*, etc, that are highly toxic, not only for microorganisms but also for host's tissues, and can lead to aberrant inflammation [22]. The relationship between the activation degree of classically activated macrophages and the inflammatory injury to the host is still poorly understood.

Here, we isolated *Ctenopharyngodon idella* macrophages from head kidney and determined the effects of *CiIFN-γ2* (*C. idella IFN-γ2*), *CiIFN-γ2* + LPS, and *CiIFN-γ2* + CS on expression of typical markers of classically activated macrophages [23,24]. We confirmed that CS + *CiIFN-γ2* enhances the activation of macrophages more strongly than *CiIFN-γ2* + LPS. Additionally, we confirmed that *CiIFN-γ2* plays a critical role in improving the ability of *C. idella* to resist *F. columnare* infection and regulate the immune function [25,26]. However, when *CiIFN-γ2* was combined with CS, it caused serious inflammatory damage and increased the mortality rate of the fish.

2. Materials and methods

2.1. Fish

The fish were obtained from the Xishui Fish Breeding Base (Huanggang, China) and acclimatised for two weeks in a recirculating freshwater system with daily feeding. The fish (120 ± 5 g) were randomly distributed into eight tanks (40 per tank). All animal experimental procedures were performed in the Fish Genetics and Breeding Laboratory in Huazhong Agricultural University. All experimental methods were performed according to the relevant guidelines and regulations.

2.2. Cloning and analysis

The open reading frame (ORF) of *CiIFN-γ2* (GenBank: JX196701) was amplified using specific primers (Table 1, *CiIFN-γ2*-F1 and –R1), designed using the Primer 5.0 program. The *CiIFN-γ2* was amplified on a ProFlex™ PCR system with the following conditions: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, and the final extension at 72 °C for 10 min. After detection on 1.0% agarose gels, all PCR products were purified from the gel using a DNA gel extraction kit (Axygen, Hangzhou, China), ligated into the pMD-18T vector (Takara, Dalian, China), and transformed into a competent *E. coli* strain Trans5α. Positive clones were manually selected and sequenced by QingKe Bioscience and Technology Company (Wuhan, China).

Table 1
Primers used for the amplification of genes and qPCR in this study.

Primer	Oligonucleotide sequence (5′-3′)
CiIFN-γ2-F1	ATGATTGCACAACACATG
CiIFN-γ2-R1	CTAAGACTCCTGCCTCTT
CiIFN-γ2-F2	ATCGGAATTCGAAAACCTGTATTTTCAGGGC AGCGTCCCTGAGAACCTG
CiIFN-γ2-R2	ATCGCTCGAGCTAAGACTCCTGCCTCTT
iNOS-F	TTCACATGGAGCACCCACAA
iNOS -R	TCAGTGCCCATGTACCAACC
IgM-F	TGGAGCAACGGCACAGTATT
IgM-R	CTCTTGGGTCTCGCACCATT
IL-1β-F	AAGTTCCCGCTTTGGAGAGTA
IL-1β-R	GCCACATACCAGTCGTTCAGT
IL-6-F	CTCAACCCTGGTCAACGACA
IL-6-R	GCATCCATGCGGATTTGACC
MHCII-F	ACAAGCCTCAGTGTGACGAG
MHCII-R	TGTGTCCGGAATCTCATGGC
STAT1-F	AGATGTTGAGCAGGTGTT
STAT1-R	TGTCTTCTAATGAGAGGG
TNF-α-F	ATTTATCTCGGTGCGGCCTT
TNF-α-R	GCTTACAGAGCAAACACCCC
β-actin-F	GCCCCACCTGAGCGTAAATA
β-actin-R	GAGTCGGCGTGAAGTGGTAA

The signal peptide of *CiIFN-γ2* was predicted using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The isoelectric points of recombinant GST-*CiIFN-γ2* fusion protein, *CiIFN-γ2* protein, and GST-tag protein were analyzed using the ExPASy Molecular Biology server (https://web.expasy.org/compute_pi/).

2.3. Expression and purification of C. idella IFN-γ2

A cDNA fragment encoding 471bp (from position 79 to 549) of the putative mature *CiIFN-γ2* (excluding the signal peptide) with an enterokinase cleavage site was obtained via PCR using specific primers (Table 1, *CiIFN-γ2*-F2 and -R2). The PCR products were cloned into the pGEX-4T1 expression vector (Novagen) after digestion using a combination of *Eco*RI and *Xho*I (Takara, Dalian, China). The confirmed recombinant expression construct (pGEX-4T1-*CiIFN-γ2*) was transformed into competent *E. coli* BL21 (DE3) (Novagen) cells. Transformed cells were induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG) at 25 °C for 5 h, after which the cells were pelleted by centrifugation (5000 × *g*) for 10 min. The cell pellet was then resuspended in 40 mL of buffer A at 25 °C (50 mM Phosphate Buffer; pH 7.5). The cells were subsequently broken by passing them through a French pressure cell press at 1.0×10^3 bar for 5 min. After centrifugation at 12,000 × *g* for 1 h at 4 °C, the protein was bound to 10 mL glutathione beads (Smart-Lifesciences, China) and eluted through a gradient of buffer B [50 mM Phosphate Buffer, 10 mM L-Glutathione reduced (pH 7.5, Temp 25 °C)]. The expressed fusion protein was validated by SDS-PAGE (Solarbio) after dialysis in the 50 mM Phosphate Buffer (PH 6.5, Temp 25 °C) overnight. The protein concentration, quantified using the BCA Protein Assay Kit (Solarbio), was adjusted to 3 mg/mL. Ten U of enterokinase (Novoprotein) was added, the sample was allowed to incubate at 16 °C for 16 h, and then detected using SDS-PAGE and Western Blot. Following that, the sample was passed through the CM Beads 6FF (Smart-Lifesciences, China) in the GE AKTA Pure to remove the GST-tags and any uncleaved proteins. The cleaved protein was then eluted until its NaCl concentration reached 490 mM. Finally, the target protein was detected using SDS-PAGE and Western Blot after dialysis overnight.

2.4. Isolation, culture and treatment of C. idella head kidney macrophages

We isolated macrophages from the head-kidney lymphocytes using their adherent growth characteristics. Macrophages were prepared using discontinuous density gradient centrifugation [27]. In brief, randomly selected six fish specimens were anaesthetized in 100 mg/L MS-222, euthanized and dissected, then head-kidney was aseptically collected, washed twice and gently pressed through a 100 μm mesh in Leibovitz medium (L-15). Cell suspension was plated in 51% percoll, and centrifuged at 400 × *g* for 30 min at 4 °C. Following this, the middle layer of cells was collected, the cells separated by a gradient of 34%/51% percoll, again centrifuged at 400 × *g* for 30 min at 4 °C, and then washed twice with PBS at 4 °C. Subsequently, the cells were diluted in 2 mL of L-15 (Boster Biological Technology, Wuhan, China) supplemented with 2% fetal bovine serum (FBS) and 50 μg/mL gentamicin, and their concentration was adjusted to 2×10^6 cells per milliliter before being seeded into four six-well plates for 4 h. In this way, highly pure macrophages were obtained. The cells were cultured for an additional 6 h in 2 mL of L-15 (10% FBS) to obtain resting macrophages.

The *C. idella* head kidney macrophages were separately stimulated by the addition of 250 ng/mL *CiIFN-γ2* protein, 250 ng/mL *CiIFN-γ2* protein + 20 μg/mL LPS (*SIgM*a), 250 ng/mL *CiIFN-γ2* protein + 20 μg/mL 20kD CS (Regent Science Industry Limited), and the same volume of

PBS as the control group. The plates were incubated at 28 °C with 5% CO_2 for 3 h, 6 h, and 12 h. At each time-point, the medium in all wells was removed and the macrophages were lysed by the addition of 1 mL Trypsin-EDTA. Lysates were stored at −80 °C until total RNA extraction. The expression levels of *IL-1β*, *TNF-α*, *IL-6*, *iNOS*, and *MHCII* were detected using qPCR. For morphological analysis, stimulated cells at the 6 h time-point were characterized using Giemsa stains [28]. In order to ensure the accuracy of the study, the tests were performed in triplicate.

2.5. Pretreatment of different groups in vivo

CiIFN-γ2 protein for the injection was prepared at the concentration of 30 μg/mL, and CS at 1.2 mg/mL. The fish were anaesthetized with 100 mg/mL tricaine methanesulfonate (MS-222) and intraperitoneally injected with 100 ng of *CiIFN-γ2* protein and/or 4 μg of CS per gram of fish weight. Fish in control group were intraperitoneally injected with isometric PBS (PH 6.5). Six hours later, these fish were submitted to a bacterial challenge with *F. columnare*.

2.6. Bacterial challenge, tissues sampling and preparation

The *F. columnare* used for the challenge was a Genomovar I strain isolated from the diseased yellow catfish (*Pelteobagrus fulvidraco*) [29]. In this study, the virulence of the *F. columnare* strain was first determined in *C. idella* challenged via an intraperitoneal injection.

After culturing the *F. columnare* in Shieh medium for 24 h at 28 °C, the CFU was measured by plate count, and then adjusted to a final concentration of 1×10^8 CFU/mL. Fishes in each group were rapidly intraperitoneally injected with 150 μL of the bacterial suspension and then gently returned to the tank. Fish in each group were monitored daily for signs of disease and survival rate recorded over the 7-day period.

Before the challenge (day 0) and 7 days post challenge, six fish from each group were anaesthetized with MS-222 and about 100 μL of serum harvested from each fish. Moreover, the spleen and head kidney were sampled (and stored at −80 °C for the RNA extraction) in order to detect the expression of immune genes by qPCR. From the batch euthanized at the day 7, we collected the whole hepatopancreas and intestines, and fixed them in 4% paraformaldehyde (PFA) for the HE staining. The viscera, comprising kidney, hepatopancreas, and spleen, were sampled and ground together for the bacterial load detection.

2.7. Bacterial load

Half a gram of ground viscera (spleen, hepatopancreas, trunk kidney) was weighted and homogenized in the Shieh medium under sterile conditions using a dounce tissue grinder (*SIgM*a). Serial dilutions (10^{-4}, 10^{-5}, 10^{-6}) were performed, 100 μL of dilution was plated onto Shieh Agar plates and incubated at 28 °C for 36 h. Finally, yellow single colonies were counted. The CFU/g was calculated taking the dilution and total weight of the viscera into consideration.

2.8. Serum biochemical parameter analysis

The blood was collected from the caudal vein, clotted at room temperature for 1 h, and then stored at 4 °C overnight. Serum was separated by centrifugation at 3000 × *g* for 10 min, and then stored at −80 °C. Lysozyme activity (LA), total superoxide dismutase (T-SOD) activity, total protein (TP) concentration and serum nitric oxide (NO) were determined using a kit (Jiancheng Bioengineering Institute, Nanjing, China).

2.9. Hematoxylin and eosin (HE) staining

The hepatopancreas and intestine were dissected and fixed immediately in 10% neutral buffered formalin for 24 h, dehydrated, paraffin-embedded, and sectioned. Sectioned samples (4 μm) were mounted on aminopropyl-triethoxysilane-coated slides. Following the deparaffinization in xylene, sections were rehydrated, stained with hematoxylin and eosin (HE), mounted with neutral gum, and the images were captured.

2.10. RNA extraction and qPCR analysis

Total RNA was extracted from spleen, head kidney, and macrophages with RNAiso Plus (Takara, China) according to the manufacturer's instructions, followed by DNAse I treatment. Total RNA was quantified by absorbance ratios at 260 nm and 280 nm using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and the quality was assessed by 1% agarose gel electrophoresis. cDNA synthesis was performed using the HiScript® II Q Select RT SuperMix for qPCR (Vazyme, China) according to the manufacturer's instructions. All the cDNA products were diluted to 250 ng/μL and qPCR performed using the AceQ® qPCR SYBR® Green Master Mix (Vazyme, China) on a Roche LightCycle® 480 System (Roche, Switzerland) according to the manufacturer's instructions. The primers for qPCR analyses are listed in Table 1 [25,30] (sequence information was obtained from http://hzaugenelab324.vicp.io/viroblast/viroblast.php). A melting curve was generated for each qPCR product to confirm the specificity of the assays, and a dilution series was prepared to check the efficiency of the reaction. *β-actin* was used as the reference gene. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on the cycle threshold (CT) values was used to analyze gene expression levels.

2.11. Statistical analysis

The results were reported as means ± SEM post data preparation and statistical analysis using GraphPad Prism 5.0 software. Statistical significance was assessed using student's two-tailed *t*-test in each experimental group relative to the control group. Significance (P-value) is indicated as: *($P < 0.05$); ** ($P < 0.01$); *** ($p < 0.001$).

3. Results

3.1. Expression and purification of C. idella IFN-γ2

E. coli BL21 (DE3) cells harboring the recombinant expression construct pGEX-4T1-*CiIFN-γ2* successfully expressed the GST-*CiIFN-γ2* fusion protein which was induced with 1 mM IPTG (Fig. 1A). The expressed products were purified using GST affinity column, and then the GST-tag was removed from the fusion protein by digestion with enterokinase, and confirmed by SDS-PAGE (Fig. 1B, expected size approximately 18 KD). Western-blot analysis was conducted using anti-GST tag antibody to detect the *CiIFN-γ2* (Fig. 1C). The results corroborate that we obtained the target protein without the GST tag.

3.2. CiIFN-γ2 protein, as well as CiIFN-γ2 protein with LPS or CS activated macrophages in vitro

The macrophage-activating potential of *CiIFN-γ2* and *CiIFN-γ2* in combination with LPS or CS was tested over a 12-h period (Fig. 2). Macrophages in the *CiIFN-γ2* and *CiIFN-γ2* + LPS groups exhibited significantly ($p < 0.05$) upregulated expression of *MHCII* and *TNF-α* at 3 h, 6 h, and 12 h (compared with the control group), maintained at a similar level during those time-points (Fig. 2D and E). However, the expression of *iNOS* exhibited a decreasing time-dependent regulation pattern (Fig. 2C). Interestingly, when macrophages were stimulated by *CiIFN-γ2* + CS, they exhibited significantly ($p < 0.05$) upregulated expression (compared with the control) of *IL-1β*, *IL-6*, *iNOS*, *MHCII*, and *TNF-α* at the 6 h time-point (Fig. 2A, B, C, D, E), followed by a downward trend. Six hours after the *CiIFN-γ2* and *CiIFN-γ2* + LPS stimulation, macrophages stained with Giemsa displayed characteristics of

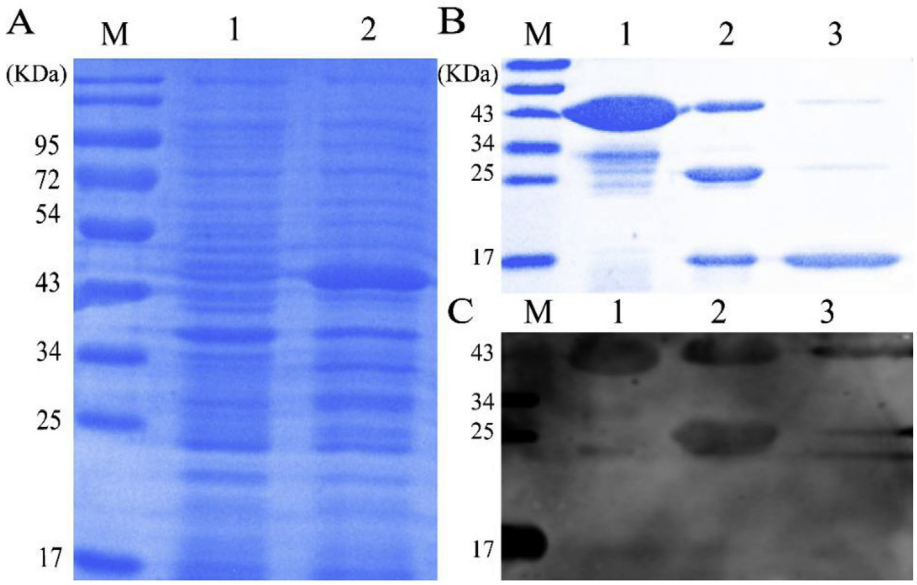

Fig. 1. (A) SDS-PAGE analysis of *CiIFN-γ2* expression. M = marker; 1 = lysate from uninduced *E.coli* cells transformed with pGEX-4T1-*CiIFN-γ2* recombinant plasmid; 2 = lysate from IPTG-induced *E. coli* cells transformed with pGEX-4T1-*CiIFN-γ2* recombinant plasmid. (B) SDS-PAGE analysis of purified *CiIFN-γ2*. M = marker; 1 = GST-tag fusion protein purification; 2 = enterokinase digestion products; 3 = purification of the target protein. (C) Western-blot identification with GST antibody detection. M = marker; 1 = GST tag fusion protein purification; 2 = enterokinase digestion products; 3 = purification of the target protein.

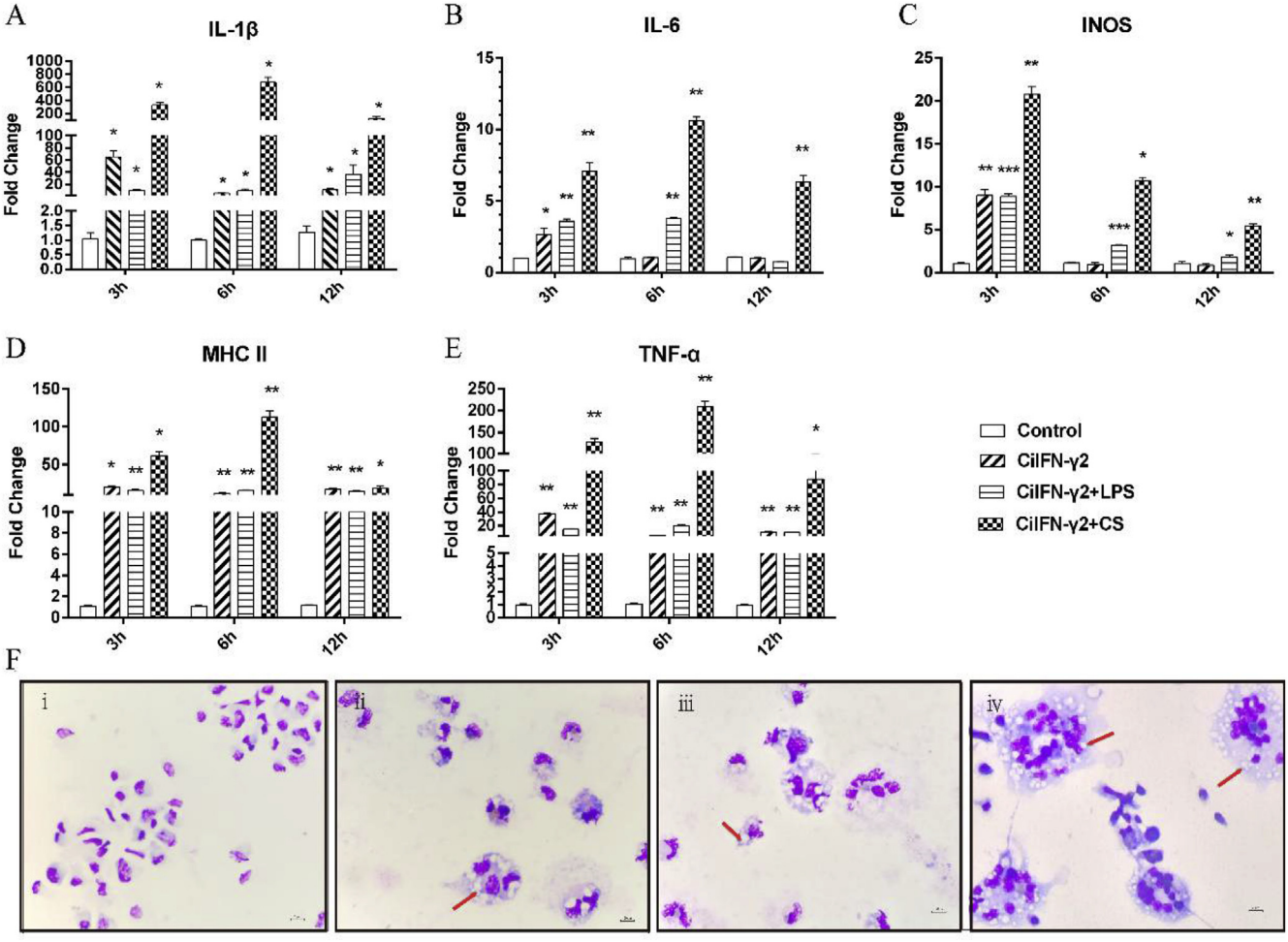

Fig. 2. Effect of *CiIFN-γ2* or *CiIFN-γ2* with LPS or CS on the immune gene expression and morphological variations of macrophages

IL-1β (A), *IL-6* (B), *iNOS* (C), *MHCII* (D), and *TNF-α* (E) expression were determined by qPCR after different stimuli. Total RNA was extracted from macrophage after 3, 6, and 12 h. All results were standardized to the levels of *β-actin*. The results are expressed as means ± SE of triplicate experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus the control group. (F) Morphological variations of macrophages stimulated with *CiIFN-γ2*, *CiIFN-γ2* + LPS, or *CiIFN-γ2* + CS for 6 h inferred using Giemsa stains. (i) PBS. (ii) *CiIFN-γ2*. (iii) CiIFN-γ2 + LPS. (iv) *CiIFN-γ2* + CS. Red arrows highlight the macrophage vacuolation (scale bar = 20 μm).

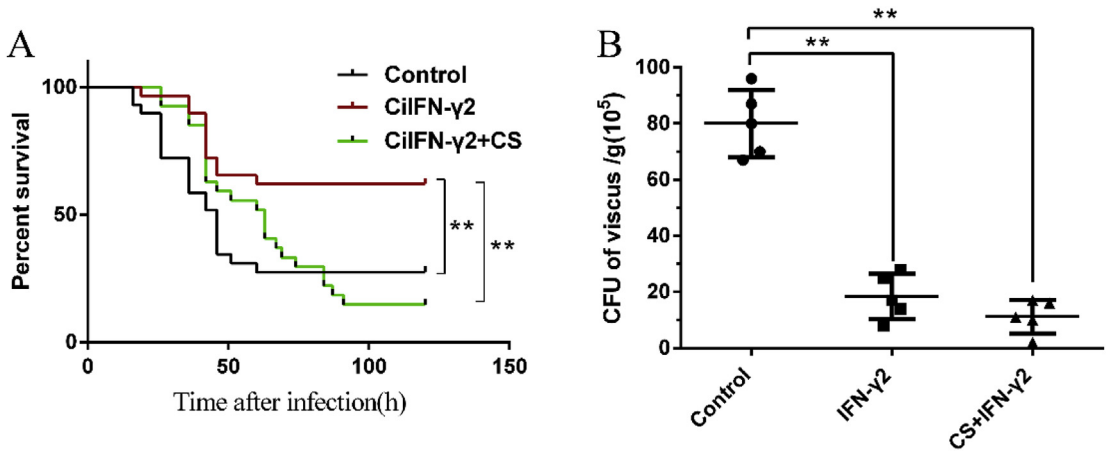

Fig. 3. Survival rates and bacterial load of *C. idella* infected with *F. columnare* in different pretreatment groups.
(A) Animals were intraperitoneally injected with *CiIFN-γ2*, *CiIFN-γ2* + CS, or PBS, and 6 h later, these fish were challenged with *F. columnare*. Mortality in each group (n = 35) was monitored during the next 7 days. (B) The viscera were sampled on the 7th day after the infection and bacterial load was detected. The results are expressed as means ± SE of triplicate experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus control.

classically activated macrophages, in that some vacuoles appeared in the cytoplasm, and lysosomes tended to form multiple pseudopods (Fig. 2F, ii, iii) [30]. Meanwhile, when stimulated by *CiIFN-γ2* + CS, macrophages exhibited a marked aggregation and a large number of vacuoles in the cytoplasm (Fig. 2F, iv).

3.3. Impacts of CiIFN-γ2 and CiIFN-γ2 + CS on the immunity of C. idella against F. columnare infection

To determine if *CiIFN-γ2* alone and *CiIFN-γ2* + CS both have the ability to enhance the *C. idella* defense against *F. columnare*, the fish were sampled 6 h before the infection. In the *CiIFN-γ2* protein-injected group, the survival rate (60%) was extremely significantly ($p < 0.01$) higher than that of the control group (27%) (Fig. 3A), and the bacterial load was significantly lower (than in the control group) (Fig. 3B). In the *CiIFN-γ2* + CS-injected group, the survival rate (13%) was not significantly different from the control group, but it was extremely significantly ($p < 0.01$) lower than in the *CiIFN-γ2* group (Fig. 3A). The bacterial load was also significantly lower than that of the control group (Fig. 3B).

3.4. The total protein, total superoxide dismutase, lysozyme activity and the nitric oxide levels in serum were increased in the CiIFN-γ2 and CiIFN-γ2 + CS groups

To analyze effects of *CiIFN-γ2* and *CiIFN-γ2* + CS on serum biochemistry, we assayed the levels of TP, LA, T-SOD, and NO (Fig. 4). Our data showed that *CiIFN-γ2* injection significantly increased the levels of TP, T-SOD, LA, and NO almost during the entire experimental period compared to the control group (Fig. 4A, B, C, D). On the other hand, the level of TP, T-SOD, and NO also increased significantly in the *CiIFN-γ2* + CS group (Fig. 4B, C, D).

3.5. Histopathological analysis

Histopathological alterations were studied in the hepatopancreas and intestine of different pretreatment groups (IFN-γ2 and IFN-γ2 + CS) 7 days after the infection with *F. columnare* (Fig. 5). In the control group, the most common abnormalities found were a messily arranged hepatocyte cord, obviously increased cell gap, vacuolar degeneration, and marginal tissue irregularity in the hepatopancreas. In the intestine, the amount of damage exhibited by the mucous layer and serous membrane (black arrows), as well as the number of goblet cells, increased in comparison to healthy fish (Fig. 5A, E). In the *CiIFN-γ2* + CS group, the most common abnormalities in the hepatopancreas were a messily arranged hepatocyte cord, increased cell gap (red arrows), vacuolar degeneration (yellow arrows), and inflamed cell infiltration (red circle). In the intestine, injury of the mucous layer, serous membrane (black arrows), and the number of goblet cells (black circle) increased obviously (Fig. 5C, G). However, in the *CiIFN-γ2* group, the severity of damage in the intestine was reduced compared with the control and *IFN-γ2* + CS groups, and there were no obvious abnormalities in the hepatopancreas (Fig. 5B, F). Fig. 5D and H panels show the hepatopancreas and intestine of healthy *C. idella*.

3.6. Relative expression of pro-inflammatory cytokines and immune-related genes in immune organs

The mRNA levels pro-inflammatory cytokines and immune-related genes in head kidney and spleen of *C. idella* at days 0 and 7 post-infection are displayed in Fig. 6. At day 0, in the spleen, *IL-6*, *iNOS*, *IL-1β*, and *STAT1* genes were significantly up-regulated in both CiIFN-γ2 and *CiIFN-γ2* + CS groups, compared with the control group (Fig. 6A). However, the up-regulation of some genes (*IL-6*, *iNOS*, *TNF-α*, and *IL-1β*) was even higher in the *CiIFN-γ2* + CS group (Fig. 6A). At day 7, *CiIFN-γ2* up-regulated the mRNA levels of *IL-6*, *TNF-α*, *MHCII*, *STAT1* and *IgM* compared with the control group (approximately 3-fold, 4-fold, 2-fold, 2-fold, 4-fold, and 8-fold, respectively; Fig. 6C). In the *CiIFN-γ2* + CS group, the expressions of *IL-6*, *iNOS*, *TNF-α*, *IL-1β*, STAT-1, and *IgM* were significantly up-regulated compared with the control group (approximately 19-fold, 2-fold, 3-fold, 16-fold, and 4-fold respectively; Fig. 6C). At day 0, in head kidney, *CiIFN-γ2* up-regulated the mRNA levels of *IL-6*, *iNOS*, *TNF-α*, *MHCII*, and *STAT1* compared with the control group (approximately 13-fold, 12-fold, 2-fold, and 3-fold respectively; Fig. 6B). All tested pro-inflammatory cytokines were significantly up-regulated in the *CiIFN-γ2* + CS group (compared to the control group) and (significantly) higher than CiIFN-γ2 group, (Fig. 6B). At day 7, *CiIFN-γ2*, and *CiIFN-γ2* + CS groups both exhibited a similar level of (significant) up-regulation of mRNA levels of *iNOS*, *TNF-α*, *IL-1β*, *MHCII,* and *IgM* (Fig. 6D).

4. Discussion

GST-tag facilitates the expression of the fusion protein in a soluble form for subsequent affinity chromatography purification [31,32], but a large molecular weight (MW) of the GST-tag affects the functioning of the *CiIFN-γ2* protein, so we chose enterokinase to digest the GST marker and obtain a CiIFN-γ2 protein of approximately 18 kD in size (Fig. 1).

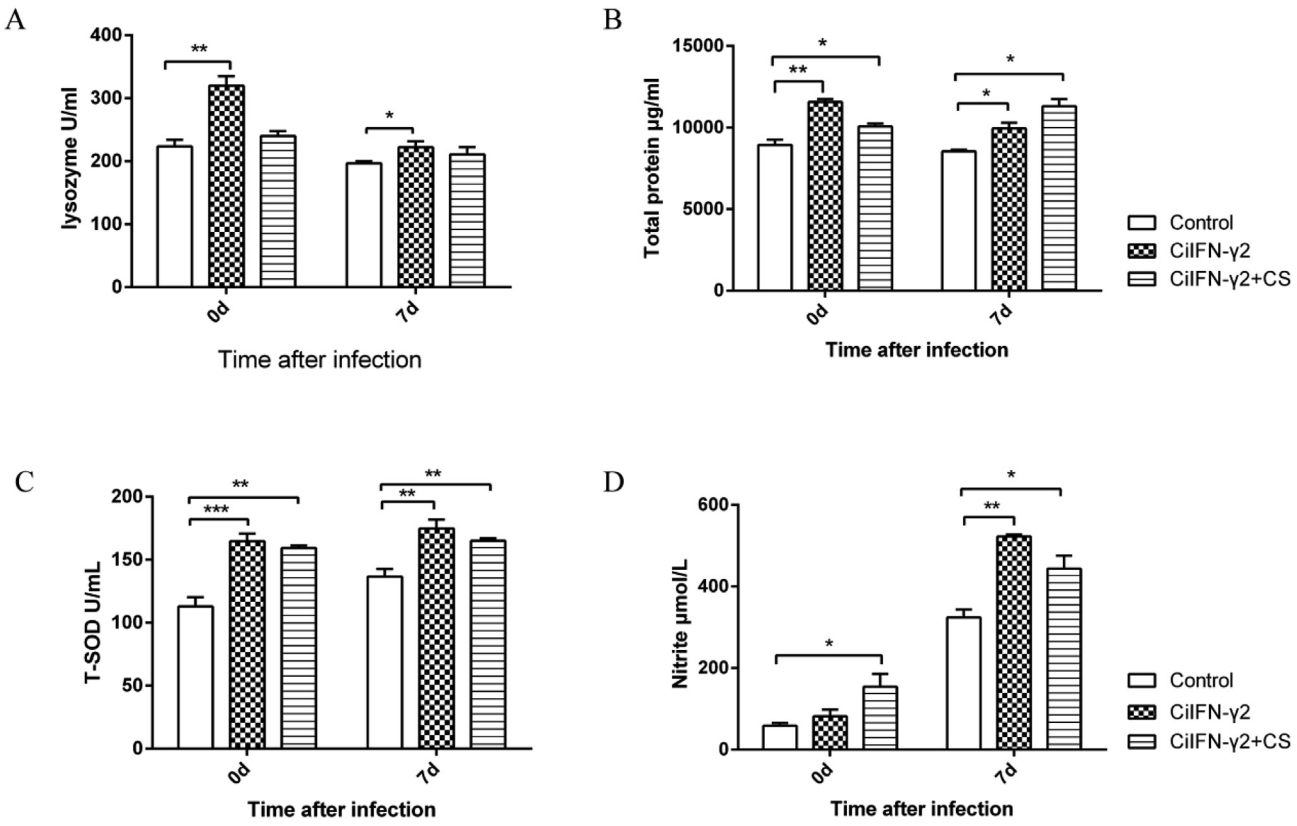

Fig. 4. Serum biochemical parameters.(A) Lysozyme activity, (B) Total protein, (C) Total superoxide dismutase, (D) Nitric oxide. These results are means ± SE (N = 6). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus the control.

In recent years, recombinant proteins of IFN-γ from goldfish, rainbow trout, and common carp have allowed *in vitro* studies on the activation of fish macrophages by IFN-γ induction [6,8,33]. For example, treatment of goldfish macrophages with recombinant IFN-γ induced increased expression of several proinflammatory genes, including *TNFα-1*, *TNFα-2*, *IL-β-1*, *IL-β-2*, *iNOS A*, and *iNOS B* [6]. Our results consistently showed that *CilFN-γ2* increased the expression levels of *IL-1β, TNF-α*, *IL-6*, and *iNOS* (a typical marker of classical activated macrophages [23,34]) in *C. idella* head kidney macrophages in a time-dependent manner (Fig. 2A, B, C, E). In mammals, a classical approach to studying inflammatory macrophages is via stimulation of the immune system with IFN-γ in combination with LPS. However, in

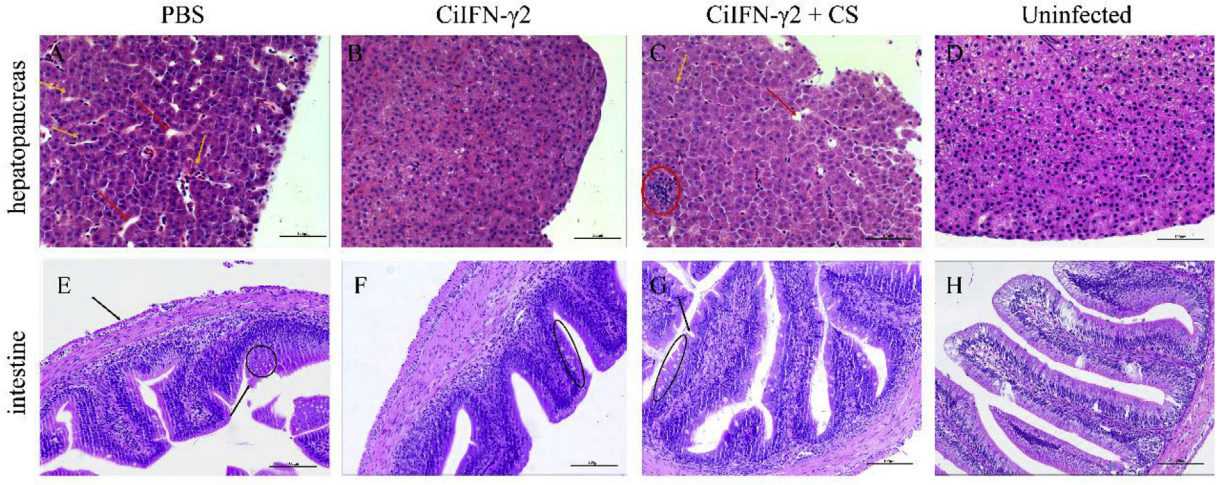

Fig. 5. Histological changes in the hepatopancreas and intestine of *C. idella* 7 days after the infection with *F. columnare* in different groups (H&E staining). (A) Hepatopancreas of the control group. Hepatocyte cord messily arranged, obviously increased cell gap (red arrows), vacuolar degeneration (yellow arrows). (B) Hepatopancreas of the *CilFN-γ2* group. No obvious histological changes in comparison to the uninfected group. (C) Hepatopancreas of the *CilFN-γ2* + CS group. Hepatocyte cord in hepatopancreas messily arranged, increased cell gap (red arrows), vacuolar degeneration (yellow arrows) and inflammatory cell infiltration (red circle). (D) Hepatopancreas of uninfected fish. (E) Intestine of the control group. Mucous layer and serous membrane injury (black arrows), and goblet cells increased (black circle). (F) Intestine of the *CilFN-γ2* group. Serous membrane injury (black arrows). (G) Intestine of the *CilFN-γ2* + CS group. Mucous layer and serous membrane injuries (black arrows), and increased goblet cells counts (black circle). (H) Intestine of uninfected fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

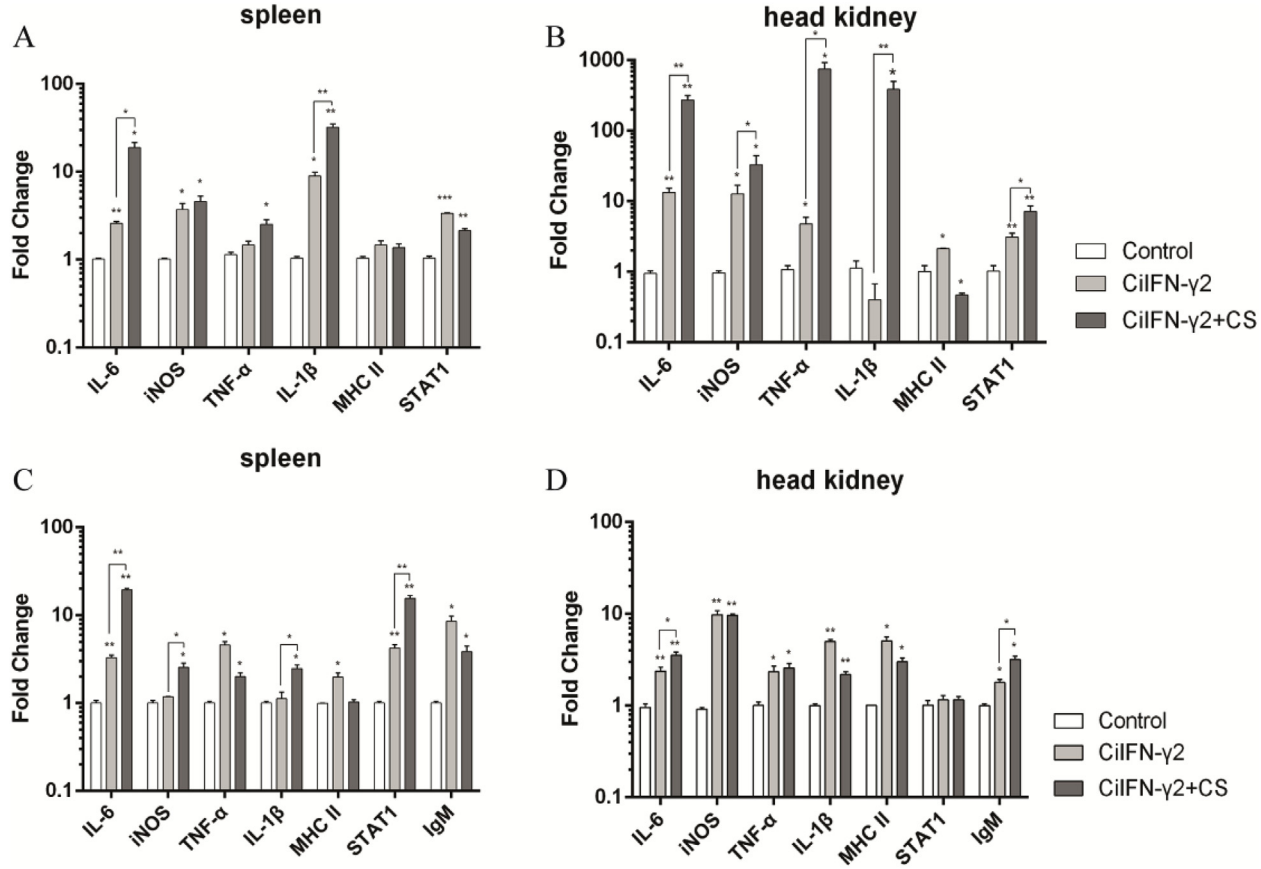

Fig. 6. Relative expression of immune-related genes in intestine, spleen and head kidney before (day 0) and 7 days after the infection. (A) spleen, day 0. (B) head kidney, day 0. (C) spleen, day 7. (D) head kidney, day 7. These results are means ± SE (N = 3). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus control.

our study, morphological variations and expression levels of *IL-1β*, *TNF-α*, and *MHCII* were very similar in macrophages of the *CiIFN-γ2* + LPS and *CiIFN-γ2* groups. This may be due to the specific LPS-recognition receptor TLR4 needing to form a tripolymer along with MD2 and the leucine-rich repeat (LRR) protein CD14 to identify the LPS. In mammals, CD14 is mainly distributed on the cell surface of monocytes, macrophages, and dendritic cells [35], but all fish genomes lack these two costimulatory molecules (MD2 and CD14) [12,13].

Chitosan is a partially deacetylated chitin (poly-β-(1 → 4)-N-acetyl-D-glucosamine), with various biological functions, including anti-microbial [36,37], anti-tumor [38,39], and immune-promoting functions [40,41]. Studies of functional effects of chitosan on macrophages in mammals produced two different hypotheses: 1. chitosan alone can significantly increase the expression of *iNOS* in macrophages [42,43]; 2. chitosan can promote the upregulation of *iNOS* in macrophages only in combination with *IFN-γ* [44,45]. The reasons for the inconsistency between these findings may be multifaceted. Firstly, the experimental periods were different, with the former two studies exposing cells to chitosan about 4–8 times longer than the latter two studies. Secondly, the form of chitosan was different between them: the former studies used water-soluble chitosan, while the latter studies used water-insoluble chitosan. As there is evidence that water-soluble chitosan has higher reactivity than water-insoluble chitosan in macrophages, we hypothesise that the former form may activate macrophages more easily [46]. Moreover, previous studies clearly demonstrated that larger MW chitosans (> 29.2 kDa) inhibited the production of pro-inflammatory cytokines (*TNF-α*, *IL-6*) induced by LPS, while smaller MW chitosans (≤ 29.2 kDa) had the opposite effect [14]. Previous studies of chitosan in fish were focused predominantly on its potential as a vaccine adjuvant or feed additive [47–50], so ours is the first study to use chitosan and *CiIFN-γ2* together to stimulate the macrophages in fish. Our results were partially consistent with the above conclusions that, in fish, water-soluble small molecule chitosan + *CiIFN-γ2* can significantly enhance the activation of macrophages in a short period, and even more strongly so than *CiIFN-γ2* + LPS.

Our study demonstrated that injection of *CiIFN-γ2* could significantly improve the survival rate of *C. idella* infected with *F. columnare* (compared with the control group). Also, though the survival rate of combined injection of *CiIFN-γ2* with CS was higher than that of the control group in the early stage of infection, eventually it reduced the protection rate of *CiIFN-γ2* to *C. idella* (Fig. 3A). Interestingly, compared with the control group, both pretreatment groups significantly reduced the bacterial load of viscera and improved the level of TP, T-SOD, LA, and NO, though the LA level in the *CiIFN-γ2* + CS group showed no significant difference (Fig. 4). Moreover, some studies showed that IFN-γ-knockout mice are highly susceptible to bacterial infection [51,52]. Specifically, peritoneal cells produce *IFN-γ* and are essential for the survival of infected mice, and bacterial clearance by these cells is impaired when *IFN-γ* secretion is reduced [53]. We hypothesised that injection of *CiIFN-γ2* improved the bacterial clearance and boosted the immunity of *C. idella.* However, most likely due to overactivation of the immune system, the challenge with *CiIFN-γ2* + CS caused excessive inflammation of the body, which ultimately led to a sharp decline in the survival rate.

In order to support our speculation, we examined the expression of inflammatory and immune genes, as well as tissue damage. Our results indicated that 6 h after the injection of *CiIFN-γ2* + CS, the expression levels of inflammatory factors were dramatically up-regulated, especially in the head kidney (Fig. 6A and B). Moreover, on the seventh day of infection, although the up-regulation levels of inflammatory factors and immune-related genes in the two treatment groups were similar, severe inflammatory damage was observed in the intestine and

hepatopancreas of the *CiIFN-γ2*+CS group, but not in the *CiIFN-γ2* group (Fig. 5). Previous research has shown that the responsiveness of macrophages *in vivo* and *in vitro* is different. A conventional approach for studying macrophage activation *in vitro* is the stimulation of cells (plated on plastic) with microbial agonists or cytokines, and the measurement of effector cytokine production and changes in gene expression [28,30]. However, *in vivo*, mature macrophages are strategically located throughout the body and perform an important surveillance function within the immune system. They constantly survey their immediate surroundings for signs of tissue damage or invading organisms, and are poised to respond by stimulating lymphocytes and other immune cells when danger signals are phagocytosed and/or detected by cell surface receptors [54]. Should the vast numbers of macrophages that inhabit the intestines, hepatopancreas, and kidney respond so readily to external stimulation, then systemic cytokine production would be continuous. Therefore, tissue macrophages, as well as newly recruited monocytes, are subject to a hierarchy of activation states that ensure that baseline tissue homeostasis is the 'default', and prevent constant inflammation [55]. This finding suggests that once this kind of tissue homeostasis is disrupted, such as by a strong stimulus like *CiIFN-γ2*+CS, the body will sustain persistent inflammation and may incur substantial damage, or even death.

In this study we identified a new model for the activation of macrophages, which could pave the way for a better understanding of the phenotypes of macrophages in fish. Here we corroborated our hypothesis that effects of classically-activated macrophages on the organism might differ depending on the level of activation, by providing evidence for the existence of a close relationship between the inflammatory damage in the organism and the activation level of macrophages. In addition, our findings suggest that *IFN-γ2* has potential as an immunopotentiator, but requires a careful selection of adjuvants.

5. Conclusions

Recombinant *CiIFN-γ2* has the ability to activate macrophages, and the water-soluble small molecule chitosan + *CiIFN-γ2* could significantly enhance the activation of macrophages, even more strongly than LPS + *CiIFN-γ2*. *In vivo*, injection with *CiIFN-γ2* protected *C. idella* from infection with *F. columnare* by improving bacterial clearance and boosting the immune system, while injection with *CiIFN-γ2*+CS reduced the protective effects of *CiIFN-γ2* due to excessive inflammation.

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Author contributions

Conceptualization, Tong Chen; Methodology, Tong Chen, Yazhen Hu, Jiancheng Zhou, Shengbiao Hu, Xun Xiao, Xiaoling Liu, Jianguo Su and Gailing Yuan; Project administration and supervision, Gailing Yuan; Validation, Tong Chen; Writing – original draft, Tong Chen; Writing – review & editing, Jianguo Su and Gailing Yuan.

Declaration of competing interest

The authors declare no competing financial interests.

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