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# Major histocompatibility complex class I (MHC Iα) of Japanese flounder (*Paralichthys olivaceus*) plays a critical role in defense against intracellular pathogen infection

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ABSTRACT

The major histocompatibility complex (MHC) is a highly polymorphic region of the vertebrate genome that plays a critical role in initiating immune responses towards invading pathogens. It is well known that MHC I molecules play a central role in the immune response to viruses. However, rare literatures were reported the role of MHC I in the resistance to intracellular bacteria. Sequences of MHC Iα were identified in multiple teleost species, including Japanese flounder (*Paralichthys olivaceus*), however, the immunological function of MHC Iα remain largely unknown. In this study, we examined the expression profile and biological activity of an MHC Iα homologue, PoMHC Iα, from *P. olivaceus*. Structural analysis showed that PoMHC Iα possesses conserved structural characteristics of MHC Iα proteins, including MHC_I domain, IGc1 domain, transmembrane region. Expression of *PoMHC Iα* was upregulated in a time-dependent manner by extracellular and intracellular bacterial pathogens and viral pathogen infection. Different expression patterns were exhibited in response to the infection of different types of microbial pathogens in different immune tissues. Recombinant PoMHC Iα increased the capability of host cells to defense against intracellular pathogen *Edwardsiella tarda* infection and enhanced the expression of immune related genes. The knockdown of *PoMHC Iα* attenuated the ability of cells to eliminate *E. tarda*, which was sustained by the *in vivo* results that overexpression of *PoMHC Iα* promoted the host defense against invading *E. tarda*. Antigen uptake assay indicated PoMHC Iα participated in cells antigen presentation. Collectively, this study is the first report that MHC Iα plays an important role in immune defense against intracellular bacterial pathogen in teleost. Taken together, these findings add new insights into the biological function of teleost MHC Iα and emphasize the importance of MHC I gene products for the control of *E. tarda* infection.

## 1. Introduction

The major histocompatibility complex (MHC) is a set of cell surface proteins, which is essential for the acquired immune system to recognize foreign molecules in vertebrates. MHC molecules bind to antigens derived from pathogens and display them on the cell surface for recognition by the appropriate T-cells [1]. MHC is a highly polymorphic region of the vertebrate genome and presents peptides to T cells leading to initiation of an adaptive immune response in many pathological conditions which plays a critical role in autoimmunity and host immune response to infection [2]. In generally, MHC genes are divided into two categories, termed class I and class II, based on the chemical structure and molecular function. The MHC class I molecule, containing a heavy chain (MHC Iα) and a non-covalently associated beta 2-microglobulin (β2m) chain, presents foreign peptide product by the degradation of intracellular pathogens to cytotoxic $CD^{8+}$ T cells [3]. The MHC I molecule is expressed on the cell membrane, where its highly variable peptide-binding domain presents peptides of approximately 9–11 amino acids in length [4]. After peptide loading, the MHC Iα molecules are presented to the cell surface for recognition by cytotoxic $CD^{8+}$ T cells, thereby triggering an immune response when the peptide originates from a non-self protein [5]. In contrast, MHC class II (MHC II)

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molecules are heterodimers that consist of an α and β chain, and are mainly expressed in the antigen-presenting cells and present processed extracellular antigens to $CD^{4+}$ T cells [6]. Traditionally, MHC I and II molecules have been thought to be responsible for the presentation of endogenous and exogenous antigens, respectively. However, MHC I molecules can also recognize and present exogenous antigens to cytotoxic T cells through a process known as cross presentation [7,8].

As MHC Iα genes have been recognized as the significant elements of the adaptive immunity in vertebrate, many MHC Iα genes have been isolated and characterized in fish. The first MHC Iα was identified in carp *(Cyprinus carpio)* [9]. Since then, more and more MHC Iα have been identified in various fish species, such as Atlantic salmon (*Salmo salar),* channel catfish (*Ictalurus punctatus*), golden pompano (*Trachinotus ovatus*), grass carp (*Ctenopharyngodon idellus*), Japanese flounder (*Paralichthys olivaceus*), large barbus (*Barbus intermedius*), medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*), red grouper (*Epinephelus akaara*), seabass (*Dicentrarchus labrax*), stickleback (*Gasterosteus aculeatus*), whitespotted bamboo shark (*Chiloscyllium plagiosum*), and zebrafish (*Danio rerio*) [9–21]. However, there are few studies on immunological function of MHC Iα in teleost.

It is well known that MHC I molecules play a central role in the immune response to viruses. However, few studies involve the role of MHC I in the resistance to intracellular bacterial pathogens. In a previous study, Srisapoome et al. reported the cloning of an MHC Iα sequence (named PoMHC Iα in this study for convenience) from Japanese flounder, *Paralichthys olivaceus*. They identified the sequence of PoMHC Iα and found that *PoMHC Iα* expression existed in multiple tissues [22]. However, the function and activity of PoMHC Iα have not been investigated. Japanese flounder is an important economic fish species widely farmed in the world, and its cultivation has been threatened by variety of pathogenic microorganisms on the invasion. In this study, expression patterns of *PoMHC Iα* upon pathogens infection were examined, and the immune response and antimicrobial activity of PoMHC Iα were investigated. This study will be helpful to further understanding of the biological functions of teleost MHC Iα.

## 2. Materials and methods

### 2.1. Fish

Clinically healthy Japanese flounder (average weight 14.4 g) were purchased from a commercial fish farm in Shandong Province, China, and maintained at 24 °C in aerated seawater. Before experiments, fish were acclimatized in the laboratory for 2 weeks and verified to be free of pathogens in the liver, kidney, and spleen, as reported previously [23]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma-Aldrich Corporation, St. Louis, MO, USA), as reported previously [24].

### 2.2. Bacterial and viral strains

*Edwardsiella tarda* and *Vibrio anguillarum* were preserved in the laboratory. *Escherichia coli* was purchased from Transgene (Beijing, China). *E. coli* S17-1λpir was purchased from Biomedal (Sevilla, Spain). Bacteria strains were cultured in Luria-Bertani broth (LB) medium at 37 °C (for *E. coli*) or at 28 °C (all other microbes). Fish infectious spleen and kidney necrosis virus (ISKNV) were kindly provided by Doctor Zhang of Qingdao Agricultural University.

### 2.3. Sequence analysis

The sequence of PoMHC Iα was obtained by PCR from flounder head kidney cDNA with primers MHCIαF1 and MHCIαR1 (Table 1). The sequence of PoMHC Iα was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART). The calculated molecular mass and theoretical isoelectric point were predicated by EditSeq in DNASTAR software package.

### 2.4. Quantitative real time reverse transcription-PCR (RT-qPCR) analysis of PoMHC Iα expression under normal conditions

RT-qPCR analysis of *PoMHC Iα* expression under normal conditions was determined as follows. Total RNA from the spleen, liver, head kidney, blood, intestine, muscle, gill, and brain were extracted using the EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). RNA was digested with DNaseI. One microgram of total RNA was used for cDNA synthesis with RevertAid Fiest Strand cDNA Synthesis Kit (Thermo Scientific, USA). RT-qPCR was performed using a Roche Lightcycler 96 (Switzerland) using the SYBR ExScript RT-qPCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) [25]. The PCR reaction was performed in a 20 μl volume containing 10 μl SYBR® premix Ex Taq™, 0.2 μM of each specific primer pairs MHCIαRTF/R (Table 1), and 2 μl diluted cDNA. The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified. The expression level of *PoMHC Iα* was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin as an internal reference [26].

### 2.5. PoMHC Iα expression upon bacterial and viral infection

RT-qPCR analysis of *PoMHC Iα* expression during bacterial infection was performed as reported previously [23]. *V. anguillarum* and *E. tarda* were cultured in LB broth at 28 °C to an optical density of 0.8 at 600 nm. Then, the cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of $5 \times 10^6$ CFU (colony forming units)/mL. ISKNV was resuspended in PBS to a concentration of $1 \times 10^6$ copies/mL. Flounder were divided randomly into four groups (16 fish per group) and injected intraperitoneally with 50 μl V. *anguillarum, E.tarda,* ISKNV, or PBS. At 6, 24, 48 and 72 h, or 1, 3, 5 and 7 d after infection, *PoMHC Iα* expression was determined by RT-qPCR in the head kidney, liver, and spleen, as described in section 2.4. The experiment was repeated three times.

### 2.6. Plasmid construction

The primers used in this study are listed in Table 1. To construct pEtPoMHCIα, which expresses a His-tagged PoMHC Iα, the sequence of PoMHC Iα was amplified by PCR with primers MHCIαF3 and MHCIαR3, and the PCR products were ligated into pEt32a at the BamH I and Hind III site. To construct pCNPoMHCIα, which expresses PoMHC Iα from the human cytomegalovirus immediate-early promoter, the coding sequence of PoMHC Iα was amplified with primers MHCIαF2 and MHCIαR2, and the PCR products were inserted into the eukaryotic expression vector pCN3 [27] at the Xho I site.

### 2.7. Protein expression and purification

Recombinant PoMHC Iα (rPoMHC Iα) was expressed and purified as descripted previously [28]. Briefly, *E. coli* BL21(DE3) was transformed with the plasmids pEtPoMHCIα; the transformants were cultured in LB medium at 37 °C to $OD_{600}$ 0.5, and expression of the exogenous protein was induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growing at 16 °C for an additional 18 h, the cells were harvested by centrifugation, and the His-tagged protein was purified under native conditions using glutathione sepharose columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified protein was dialyzed in phosphate-buffered saline (PBS) and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein

**Table 1**
Primers used in this study.

| Primers name | Sequence(5′–3′) | Gene accession number |
|---|---|---|
| MHCIαF1 | ATGCACACTTTACTTTTCCT | BAD13368.1 |
| MHCIαR1 | TTGTGGGTTCAGAGGCTTT | |
| MHCIαRTF | TGACGGGACCTTCCAGATGA | |
| MHCIαRTR | ATGACAGCGAGGACGACAGC | |
| MHCIαF2 | CTCGAGATGAGGACTCACTCTCTGAAGTATTT (Xho I) | |
| MHCIαR2 | CTCGAGTTGTGGGTTCAGAGGCTTT (Xho I) | |
| MHCIαF3 | GGATCCATGAGGACTCACTCTCTGAAGTAT (BamH I) | |
| MHCIαR3 | AAGCTTTTGTGGGTTCAGAGGCTTT (HindIII) | |
| siPoMHCIα-F1 | GATCACTAATACGACTCACTATAGGGGGAACGCTTCAACCAGACTTT | |
| siPoMHCIα-R1 | AAAGTCTGGT TGAAGCGTTC CCCCTATAGT GAGTCGTATT AGTGATC | |
| siPoMHCIα-F2 | AAGGAACGCTTCAACCAGACTCCCTATAGTGAGTCGTATTAGTGATC | |
| siPoMHCIα-R2 | GATCACTAATACGACTCACTATAGGGAGTCTGGTTGAAGCGTTCCT | |
| siPoMHCIα-C-F1 | GATCACTAATACGACTCACTATAGGGGAGCGAATCTACGACTCCATT | |
| siPoMHCIα-C-R1 | AATGGAGTCGTAGATTCGCTCCCCTATAGTGAGTCGTATTAGTGATC | |
| siPoMHC Iα-C-F2 | AAGAGCGAATCTACGACTCCACCCTATAGTGAGTCGTATTAGTGATC | |
| siPoMHCIα-C-R2 | GATCACTAATACGACTCACTATAGGGTGGAGTCGTAGATTCGCTCTT | |
| β-actinRTF | GGACATCCGTAAGGACCTGT | XM_020103099.1 |
| β-actinRTR | GCCTCCGATCCATACAGAGT | |
| CD8α-RT-F | GCACCTACARCTGCACGAT | BAC66490.1 |
| CD8α-RT-R | CCAATTCTAAAATACTGAACATCC | |
| IL-10RT-F | GAGTGACGGAGGAAACCAAGG | XP_019942117.1 |
| IL-10RT-R | CCGTCTAGAGGCCAGGTATTTCTC | |
| TNFα-RT-F | CACAGGGTATGGCTCTTCACG | AB040448.1 |
| TNFα-RT-R | GCCCAGGTAGATGGCATTGTA | |
| IFNγ-RT-F | ATCGCTTCAAGGAGCAGAGC | AB435094.1 |
| IFNγ-RT-F | TTTGACCTTCCGAGCTTGTCT | |
| TCRα-RT-F | GGTCTGATGCTTCACAGTGAG | KY973632.1 |
| TCRα-RT-F | ACCGCCGGATCTTTCTTCA | |

was analyzed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of purified protein was determined using the Bradford method with bovine serum albumin as the standard.

### 2.8. Effect of rPoMHC Iα on bacterial infection

The experiment was performed as described as before [29]. Head kidney lymphocytes (HKLs) were prepared using discontinuous density of Percoll solution [30]. *E. tarda* was cultured to mid-logarithmic phase, washed, and resuspended in L-15 medium to $5 \times 10^6$ CFU/mL. HKLs ($1 \times 10^5$ cells/well) were added with different concentrations of rPoMHC Iα (5, 10, 20, 30 μg/mL) or PBS, and incubated at 23 °C for 2 h. After the incubation, the cells were mixed with *E. tarda*. The plate was incubated at 23 °C for 6 h and cells were washed three times with PBS. The cells were lysed and the number of bacteria was obtained by plate count. The assay was performed three times.

### 2.9. Effect of rPoMHC Iα on expression of immune related genes

After incubating with rPoMHC Iα or PBS for 2 h, the HKLs were mixed with *E. tarda* for 6 h as described above. Then the cells were washed three times with PBS. Total RNA was prepared and used for RT-qPCR analysis of IFNγ, IL-10, TNFα, TCRα, and CD8α and PoMHC Iα expression as described above. The assay was performed three times.

### 2.10. In vivo effect of PoMHC Iα overexpression on bacterial infection

Flounder were administered with pCNPoMHCIα, pCN3, or PBS (control) by intramuscular injection. At 5 d after plasmid administration, the fish were infected with *E. tarda* as described above. Kidney and spleen were taken under aseptic conditions at 24 h and 48 h post-infection. Bacterial number in the tissues was determined by plate count [31]. Briefly, the tissue was homogenized in PBS and the homogenate was diluted serially, then the dilutions were plated on LB agar plates. The plates were incubated at 28 °C for 48 h, and the colonies that appeared on the plates were enumerated. The experiment was performed three times.

### 2.11. PoMHC Iα knockdown and its effect on bacterial infection

Knockdown of PoMHC Iα was achieved via injection of synthesized siRNA (PoMHCIα-Ri). siRNA interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation. The siRNA was synthesized with an In vitro Transcription T7 Kit (for siRNA Synthesis) (TaKaRa Biotechnology Co., Ltd., Dalian, China). In brief, two pairs of primers, siPoMHCIα-F1/R1 and siPoMHCIα-F2/R2 (Table 1), which contained the target sequence plus the T7 RNA Polymerase promoter sequence and 6 extra nucleotides upstream of the minimal promoter sequence, were designed to obtain two DNA oligonucleotides after incubation at 95 °C for 2 min. Then, the templates were allowed to cool to 25 °C during 45 min and maintain 10 min. Next, the two DNA oligonucleotides were used to transcript in vitro at 42 °C for 2 h following the manufacturer's instructions. Afterwards, the DNA template was removed from the separate short RNA strands by digestion with DNase. Finally, the synthesized siRNA was purified following the manufacturer's instructions. The control siRNA (PoMHCIα-RiC) was synthesized with two pairs of primers siPoMHCIα-CF1 and R1 and siPoMHCIα-CF2 and R2 (Table 1) as given above.

Transfection was performed as reported previously [31]. Briefly, HKLs were distributed into two 96-well culture plates ($1 \times 10^5$ cells/well) in L-15 medium without FBS. Transfection of the cells with PoMHCIα-RiC, PoMHCIα-Ri and PBS were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions given by the manufacturer. After transfection for 24 h, plates were replaced with new medium containing $1 \times 10^6$ *E. tarda*. The cells were incubated at 23 °C for 6 h and washed three times with PBS. The bacterial number was determined as described above. The cells not treated with *E. tarda* were used to isolate the total RNA as described. The assay was performed three times.

### 2.12. Antigen uptake assay

PoMHC Iα overexpression was performed as described above. Blood was collected from caudal veins of pCNPoMHCIα treated fish and control fish, peripheral blood leukocytes (PBL) were prepared with Percoll, as reported previously [30]. Antigen uptake was measured in PBL as reported by Lee [34]. Briefly, FITC labeled-chicken OVA (FITC-OVA) (Sigma) was used as the model Ag. PBL prepared above were resuspended in PBS to $1 \times 10^6$ cells, PBL were then treated with FITC-OVA (10 μg/mL) for 4 h at 22 °C. Uptake was terminated by washing the cells with ice-cold PBS. Experiments were also conducted on ice to inhibit intracellular uptake as a control for surface binding of the antigen. Fluorescence was acquired on a FACSAria (BD Biosciences) flow cytometer and analyzed using the FlowJo Software (Tree Star).

Flounder PBLs were prepared and were incubated with 2.5 μM CFSE (Molecular Probes) in complete medium for 15 min at 22 °C. CFSE staining was stopped by adding 5%BSA and cells was washed twice with complete medium. Cells were incubated with OVA (25 μg/mL) or with OVA and rPoMHCI α (10 μg/mL) in 22 °C for 24 h, then washed twice with PBS by centrifugation. Finally, cells were analyzed by flow cytometer.

### 2.13. Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA) followed by Tukey post-hoc test using a $p < 0.05$ level of significance.

## 3. Results

### 3.1. The sequence of PoMHC Iα

PoMHC Iα is composed of 353 amino acid residues with a calculated molecular mass of 40.2 kDa and a theoretical pI of 4.87. PoMHCIα contains a signal peptide sequence (residues 1 to 18), an MHC_I domain (residues19 to 195), an immunoglobulin C-Type (IGc1) domain (residues 211 to 285), and a transmembrane region (residues 307 to 329) (Fig. S1). A potential N-linked glycosylation site and protein kinase C phosphorylation site appeared in the MHC_I domain. cAMP- and cGMP-dependent protein kinase phosphorylation site appeared in IGc1 domain. The PoMHCIα has four highly conserved cysteine residues, and two are in the MHC_I domain while another two are in IGc1 domain. These conserved cysteines are capable of forming the characteristic immunoglobulin domain disulfide bonds. BLAST analysis showed that PoMHC Iα shares 59.60–73.15% overall sequence identities with MHC Iα of a number of teleost species including *Verasper variegatus, Trachinotus ovatus, Monopterus albus, Oryzias latipes, Dicentrarchus labrax, Scatophagus argus,* and *Sparus aurata* (Fig. S2). PoMHC Iα also shared 27.99% overall sequence identity with human MHC Iα. Phylogenetic analysis showed that PoMHC Ia was classified into U lineage by phylogenetic analysis (Fig. S3). Three alpha domain of U lineages, α1 domain (residues 19 to 105),an α2 domain (residues 106 to 198),and an α3 domain (residues 199 to 291) were existed in PoMHC Iα (Fig. S2).

### 3.2. Expression of PoMHC Iα under different conditions

Under normal physiological conditions, RT-qPCR analysis showed that *PoMHC Iα* expression distributed in all the examined tissues, and with an increasing order, in intestine, heart, spleen, head kidney, gill, liver, and blood (Fig. 1).

To examine the expression patterns of the *PoMHC Iα* upon microbial infection, flounder were challenged experimentally with the intracellular bacterial pathogen *E. tarda*, the extracellular bacterial pathogen *V. anguillarum*, and the viral pathogen ISKNV. At 6, 24, 48, and 72 h post-bacterial infection (hpi) or 1, 3, 5, and 7 d post-viral infection

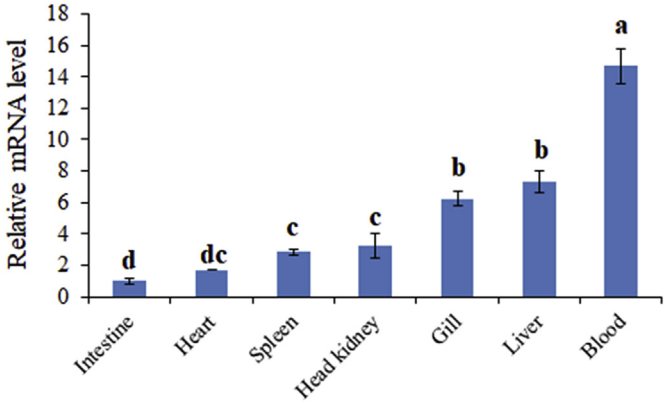


**Fig. 1. *PoMHC Iα* expression in fish tissues under normal physiological conditions**. *PoMHC Iα* expression in the intestine, heart, spleen, head kidney, gill, liver, and blood of flounder were determined by quantitative real time RT-PCR. The expression level of *PoMHC Iα* in intestine was set as 1. Data are presented as means ± SEM. Means with different letters differ significantly (Tukey's test, $\alpha = 0.05$, a > b > c > d).

(dpi), the expressions of the *PoMHC Iα* in the spleen, head kidney, and liver of the infected fish were determined by RT-qPCR. The results showed that expression patterns of *PoMHC Iα* in a manner depended on the nature of the pathogen, tissue type, and infection time. Specifically, upon the infection of *V. anguillarum*, the expression of *PoMHC Iα* in head kidney was significantly increased at 6 and 24 hpi with peaked expression at 24 hpi (4.99-fold), and fell back to the normal level at 48 hpi; the expressions of *PoMHC Iα* in spleen and liver were significantly upregulated at all the examined times, with peaked expression at 24 hpi (8.85- and 10.03-fold, respectively) (Fig. 2).

After *E. tarda* infection, *PoMHC Iα* expression in head kidney was significantly upregulated at 6, 24, and 48 hpi, with peaked expression at 24 hpi (7.78-fold); *PoMHC Iα* expression in spleen was significantly upregulated all the examined times, with peaked expression at 48 hpi (5.97-fold); Except 6 hpi, the expression of *PoMHC Iα* in liver significantly induced at other three times, with the maximum induction occurring at 48 hpi (6.25-fold) (Fig. 2).

Upon infection of ISKNV, the expression of *PoMHC Iα* in head kidney remarkably enhanced at 1 dpi (21.71-fold), then fell back to the normal level. Similar with the results in head kidney, *PoMHC Iα* expression in spleen and liver was remarkably regulated at 1 dpi (14.38- and 18.04-fold, respectively), then fell down relatively low level (Fig. 2). The aforementioned results indicated that *PoMHC Iα* could participate in the anti-infectious immunity of Japanese founder.

### 3.3. In vitro potential of rPoMHC Iα against bacterial infection and its effect on immune-related genes expression

Since PoMHC Iα was involved in defense against pathogen, we want to know whether PoMHC Iα have any effect on immune cell activity. To detect this opinion, rPoMHC Iα was purified using nickel nitrilotriacetic acid columns, and SDS-PAGE showed that the purified proteins appeared as a single band with the predicted molecular masses (Fig. S4). Head kidney lymphocytes (HKLs) of flounder were extracted and cultured in L-15 medium. To determine the effect of rPoMHC Iα on immune cell activity, HKLs were incubated with different concentrations of rPoMHC Iα. Then HKLs were infected with *E. tarda*, and the bacteria infected with the cells were enumerated. The results showed that numbers of *E. tarda* recovered from cells treated by rPoMHC Iα were enormously lower than those of control, which indicated that rPoMHC Iα played a role in defense against *E. tarda* infection (Fig. 3).

To detect whether rPoMHC Iα have any effect on the expressions of cytokines, RT-qPCR was performed after HKLs treated by *E. tarda* or by rPoMHC Iα and *E. tarda*. The results showed that rPoMHC Iα

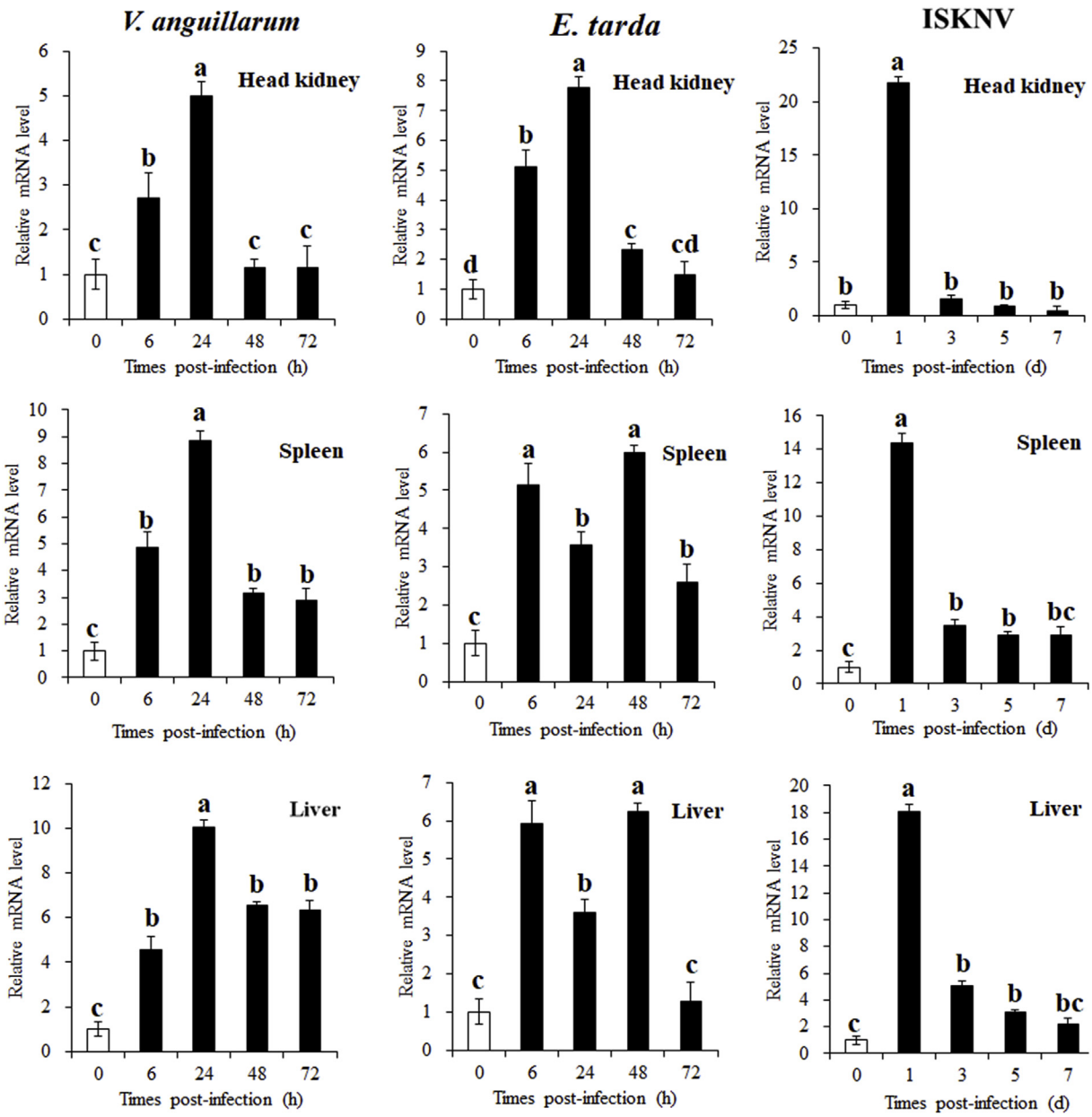


**Fig. 2. *PoMHC Iα* expression in response to pathogens challenge.** Japanese flounder were infected with *Vibrio anguillarum*, *Edwardsiella tarda*, or ISKNV (PBS as the control). The *PoMHC Iα* expressions in head kidney, spleen, and liver were determined by quantitative real time RT-PCR at various time points. In each case, the expression level of the control fish was set as 1. Data are presented as means ± SEM. Means with different letters differ significantly (Tukey's test, $\alpha = 0.05$, a > b > c > d).

significantly enhanced the expressions of IFNγ, IL-10, TNFα, TCRα, and CD8α. We also found that the expression of *PoMHC Iα* was enormously induced by rPoMHC Iα (Fig. 4). These results suggested that rPoMHC Iα possessed the immunoregulatory properties.

### 3.4. Overexpression of the PoMHC Iα in flounder and its effect on pathogen infection

#### 3.4.1. Overexpression of PoMHC Iα

Since, as observed above, *PoMHC Iα* could participate in the immune defense response of flounder to pathogen infection, we further investigated the in *vivo* effect of *PoMHC Iα* overexpression on host defense against bacterial pathogens. To make *PoMHC Iα* overexpress in flounder, the eukaryotic expression plasmid pPoMHCIα was constructed, which constitutively expresses *PoMHC Iα*. Japanese flounder were administered with pPoMHCIα or the control plasmid pCN3 by intramuscularly injection. At 5 d post-administration, distribution of the plasmids was checked by PCR and the expression of the MHC Iα was examined by RT-qPCR. The results showed that PCR detected pPoMHCIα in the muscle, kidney, and spleen of the fish administered with pPoMHCIα plasmids, but not in the fish administered with pCN3 and PBS (Fig. S5 and data not shown). It is well verified that pCN3 and recombinant plasmids based pCN3 can exist steadily and express in flounder and other species of fish [32–35]. RT-qPCR showed that the expression of MHCIα in the kidney and spleen of pPoMHCIα-administered fish was significantly higher than that of pCN3- or PBS- administered fish (Fig. 5). These results indicated that the *PoMHC Iα* gene carried on pPoMHCIα was successfully expressed in fish tissues.

#### 3.4.2. Effect of PoMHC Iα overexpression on pathogen infection

To examine whether overexpression of PoMHC Iα had any impact on bacterial infection, the fish were infected with *E. tarda* at 5 d post-

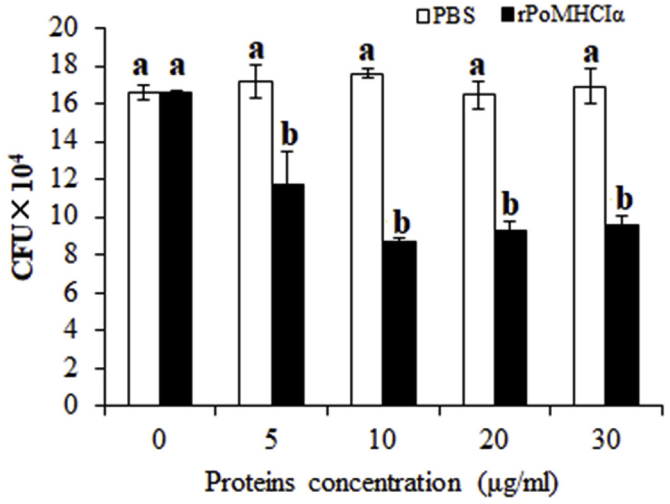


**Fig. 3. Effect of rPoMHC Iα on bacterial infection.** Head kidney lymphocytes (HKLs) were incubated with different concentrations of rPoMHC Iα or PBS (control) for 2 h, then the HKLs were infected with *Edwardsiella tarda* for 6 h, and the amounts of bacteria were determined by plate count. Data are presented as means ± SEM. Means with different letters differ significantly (Tukey's test, $\alpha = 0.05$, a > b).

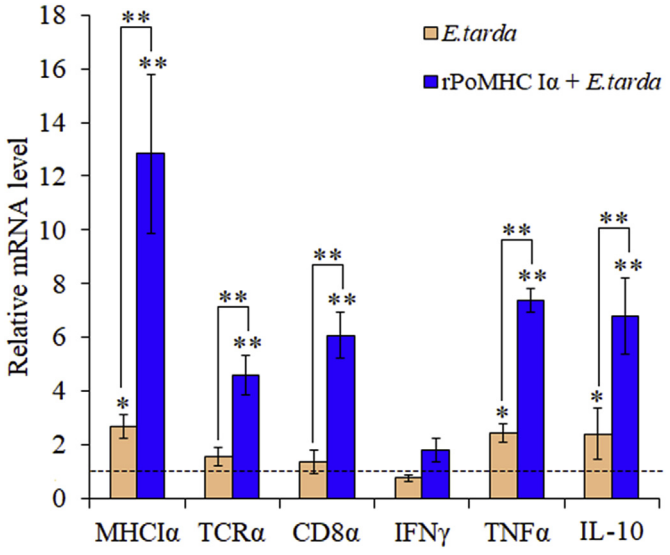


**Fig. 4. Effect of rPoMHC Iα on the expression of immune related cytokines.** Head kidney lymphocytes (HKLs) were incubated with PBS or rPoMHC Iα for 2 h, then HKLs were mixed with *E. tarda* for 6 h. The cells were wash three times with PBS. Total RNA was prepared from the cells, and used for RT-qPCR analysis of IFNγ, IL-10, TNFα, TCRα, CD8α, and PoMHC Iα expression. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$; **, $P < 0.01$.

plasmid administration, and bacterial numbers in spleen and kidney were determined at 24 h and 48 h post-infection. The results showed that in spleen, the bacterial numbers in pPoMHCIα-administered fish were significantly lower than those in the control fish at both time points, whereas the bacterial numbers in pCN3-administered fish were comparable to those in the control fish (Fig. 6A). Similarly, in kidney, the bacterial numbers in pPoMHCIα-administered fish were significantly reduced compared to those in the control fish or in pCN3-administered fish (Fig. 6A). Meanwhile, the mortality of fish infected by *E. tarda* was examined after infection. The results showed that the accumulated mortality of pPoMHCIα-administered fish was significantly lower than those of control fish or in pCN3-administered fish (Fig. 6B).

We also found *PoMHC Iα* overexpression induced the expression of immune related genes, such as TNFα, CD8α, and IL-10 (data not shown).

### 3.5. PoMHC Iα knockdown and its effect on antibacterial in HKLs

To further verify the function of PoMHC Iα, we examined the effect of *PoMHC Iα* knockdown on bacterial invasion. For this purpose, PoMHCIα-Ri and PoMHCIα-RiC (RNAi control) were synthetized and transfected into head kidney lymphocytes (HKLs). The expression of *PoMHC Iα* in the HKLs was determined by RT-qPCR and the results showed that in *PoMHCIα*Ri-administered cells, the expression of *PoMHC Iα* was significantly reduced compared to that in the control cells. However, the expression of in *PoMHCIα*RiC-administered cells was comparable to that in PBS-administered cells (NC) (Fig. 7A). To examine the effect of *PoMHC Iα* knockdown on bacterial infection, HKLs treated with PoMHCIα-Ri or PoMHCIα-RiC were infected with *E. tarda*, and bacterial numbers were determined at 6 h post-infection. The results showed that cells administered with PoMHCIα-Ri exhibited significantly increased bacterial amounts compared to control cells, whereas cells administered with PoMHCIα-RiC exhibited bacterial amounts similar to those in the control cell (Fig. 7B).

### 3.6. Antigen presentation of PoMHC Iα

To examine whether PoMHC Iα participated in antigen-presentation, flounder were administered with pCNPoMHCIα and PBL were collected. After incubating with FITC-OVA, cells were determined by FACS. The results showed that compared control cells, pPoMHCIα overexpressed cells exhibited a significantly higher amount of positive cells (M1 of 41.7% vs. 7.1%) (Fig. 8). To detect cells proliferation effect of PoMHCI α, PBLs stained by CFSE were stimulated with OVA or with OVA and rPoMHCI α. FCM analysis showed rPoMHC Iα enhanced the ability of PBLs proliferation, at the same time, the fluorescence intensity of PBLs was attenuated (Fig. 9). These results suggested that the present of PoMHC Iα enhanced the capacity of cells antigen presentation.

## 4. Discussion

MHC I genes play an important role in the immune response of vertebrates. In this study, we analyzed a major histocompatibility complex orthologue, PoMHC Iα, from Japanese flounder, and examined its expression and biological function. Structural analysis showed that PoMHC Iα possesses conserved structural characteristics of MHC Iα, including MHC_I domain, IGc1 domain, transmembrane region. N-linked glycosylation site is also found in the MHC_I domain. Similarly, some other teleost species were found to have one or more glycosylation site, such as sea bass (*D. labrax*), golden pompano (*T. ovatus*), and stickleback (*G. aculeatus*) [15,19,20]. Four conserved cysteine residues among fish and human, which are capable of forming the characteristic immunoglobulin domain disulfide bonds [36], were also found in PoMHC Iα. The amino acid sequence of PoMHC Iα has high identities with MHC Iα homologues of teleosts and shares moderate identities with MHC Iα isoforms of human and mice. These similarity of sequence and conserved structure suggested that higher and lower vertebrates share conservative function, and that different kinds of fish share alike function.

In both mammals and teleosts, MHC Iα are known to be distributed in a wide range of tissues. In golden pompano, relative high expression of MHC Iα was found in the intestine, head kidney, gill and spleen [20]. In grass carp, spleen and gill were the rich tissues of MHC Iα [13]. In our study, *PoMHC Iα* was ubiquitously expressed in all detected tissues, with higher expression in the blood, liver, gill, head kidney, and spleen, and lower expression in the heart and intestine, which is also slightly different with previous report [22]. The discrepant expression patterns may result from the difference in the physiological status of the fish,

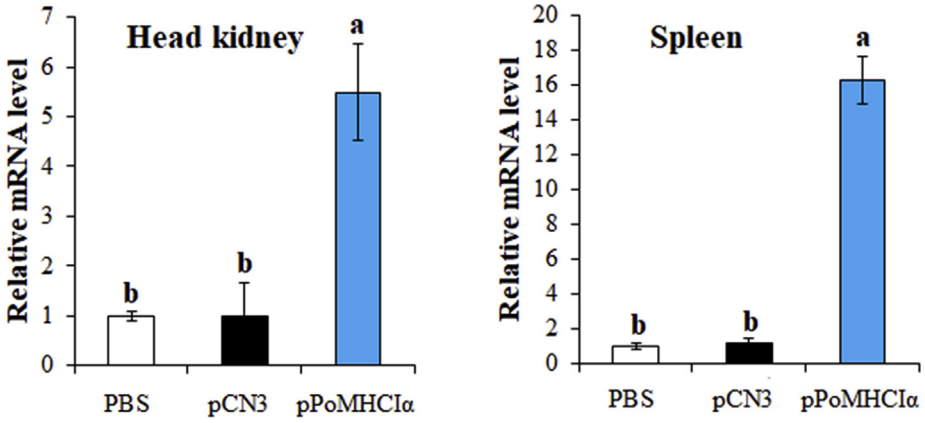


**Fig. 5. RT-qPCR analysis of PoMHCIα overexpression in Japanese flounder**. Flounder were administered with pPoMHCIα, pCN3, and PBS. At 5 days plasmid administration, RNA was extracted from spleen and head kidney and used for RT-qPCR with primers specific to PoMHCIα, or, as an internal control, β-actin. Data are presented as means ± SEM. Means with different letters differ significantly (Tukey's test, α = 0.05, a > b).

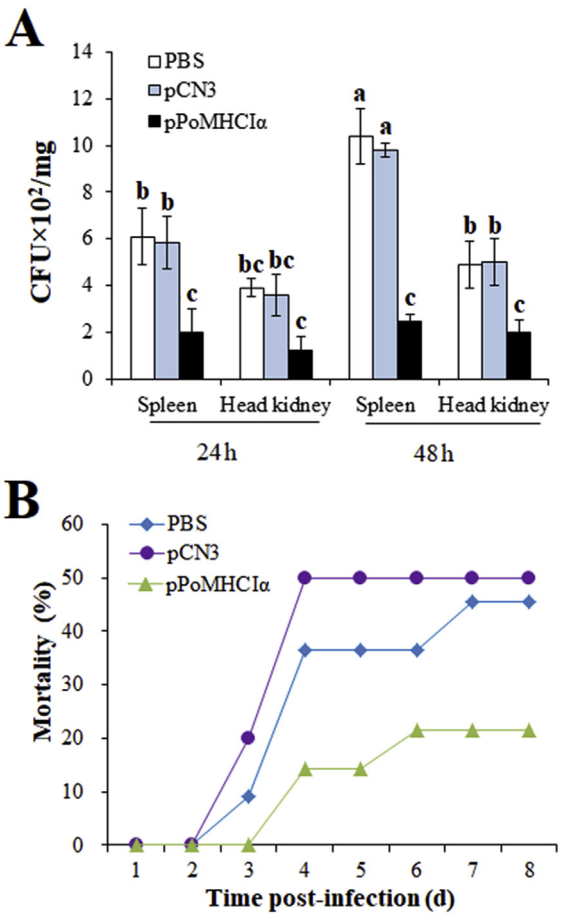


**Fig. 6. Effect of *PoMHC Iα* overexpression on bacterial infection**. Flounder were administered with pPoMHCIα, the control plasmid pCN3, or PBS (control). After 5 days post-plasmid administration, the fish were infected with *E. tarda*. Bacterial amounts in kidney and spleen were determined at 24 h and 48 h post-infection (A), and accumulated mortality were monitored for a period of 15 days (only 8 days are shown since no more deaths occurred after 8 days) (B). Data are presented as means ± SEM. Means with different letters differ significantly (Tukey's test, α = 0.05, a > b > c).

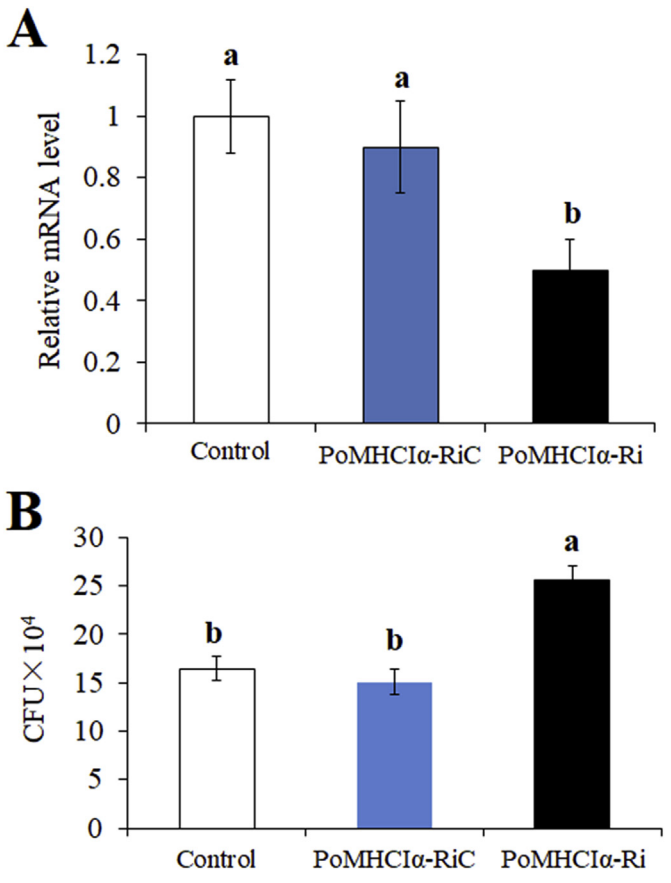


**Fig. 7. Effect of *PoMHC Iα* knockdown on bacterial infection.** PoMHCIα-Ri and PoMHCIα-RiC (RNAi control) were synthetized and transfected into Japanese flounder head kindye lymphocytes (HKLs), the expression of *PoMHC Iα* was determined by RT-qPCR (A). After transfecting, the HKLs were infected with *Edwardsiella tarda* for 6 h, and the amounts of bacteria were determined (B). Control, the cells treated with PBS. Data are presented as means ± SEM. Means with different letters differ significantly (Tukey's test, α = 0.05, a > b).

which can vary with age, culture condition, and fish species, and from the difference in experimental conditions. It is well known that the head kidney and spleen are the main immune organs in fish [37]. *PoMHC Iα* expressed highly in these two tissues suggested that both of the organs may involve in the immune system in flounder. As gill is often exposed to the external environment and contacts with pathogens [8], the high expression of *PoMHC Iα* in gill indicated their immune functions against infection. These results suggested that the head kidney, spleen, and gill play important roles in the adaptive immune response through MHC class I pathway in *P. olivaceus*.

Increasing reports showed that the expression of MHC Iα was upregulated by pathogens infection [3,20,38–40]. For example, challenge of blunt snout bream with extracellular pathogen, *Aeromonas hydrophila*, resulted in a significant increase in the expression of MHC I within 72 h after infection in gill, kidney, intestine and liver [3]. Infection by virus such as IHNV, ISAV, SGIV also upregulated the

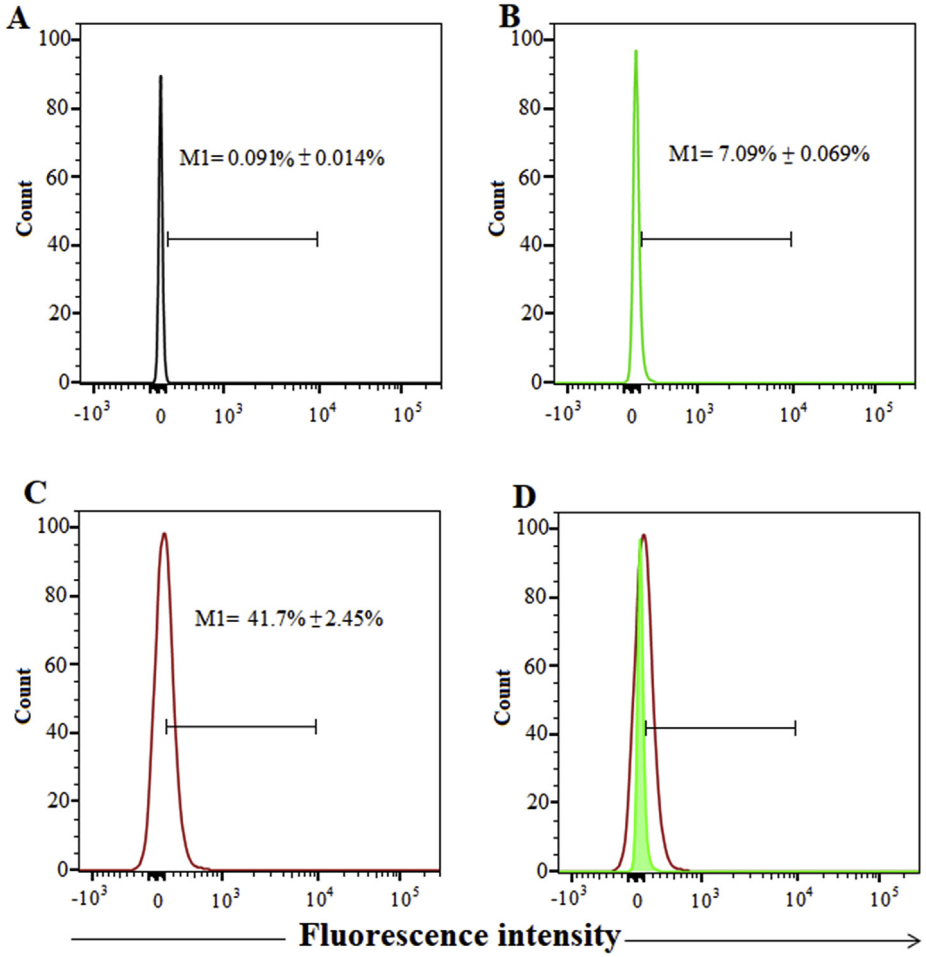


**Fig. 8. Analysis of PoMHC Iα antigen presentation.** PBLs were collected from PoMHC Iα overexpressed fish and control fish, respectively. Then cells were incubated with FITC-OVA and analyzed by fluorescence activated cell sorting. A, PBLs were incubated with PBS. B, PBL were incubated with FITC-OVA. C, PoMHC Iα overexpressed PBLs were incubated with FITC-OVA. D, merged images of B and C. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed.

expression of MHC class I in different fish species [41–44]. Consistent with these results, our study showed that the expression of *PoMHC Iα* was significantly induced by the challenge of extracellular bacterial pathogen *V. anguillarum*, the intracellular bacterial pathogen *E. tarda*, and the viral pathogen ISKNV. The maximum induction by ISKNV was higher than that by bacterial pathogen *V. anguillarum* and *E. tarda*. These results suggested that PoMHC Iα not only participated in the presentation of endogenous antigens, but also was involved in presentation of exogenous antigens, which supported the opinion that MHC I molecules can also recognize and present exogenous antigens to cytotoxic T cells [7,8,45]. Conversely, some viral infections resulted in down-regulation of MHC class Iα. For example, mouse Norovirus infection reduces the surface expression of MHC class Iα [46]. MHC class Iα expression in intestinal cells is reduced by rotavirus infection [47]. Challenge of carp with virus KHV lead to down-regulation of MHC class Iα mRNA expression [48]. In VHS-infected olive flounder, the MHC class Iα expression was high at early stage of infection (3–6 hpi) but expression reduced below control levels at later stages (2–7 dpi) [44]. The above findings indicated that upon pathogen infection, expression pattern of MHC class I appeared pathogen-dependent and time-dependent, which is closely related to its immunologic function.

It is well known that MHC I molecules play a central role in the immune response to viruses. Studies showed that MHC Iα promoted the control of both HIV and simian immunodeficiency virus (SIV) replication by activating $CD^{8+}$ T-cell populations [4]. Chicken MHC haplotypes encoding promiscuous class I molecules can confer protection against a variety of viral infections under experimental and field conditions [49]. To achieve the purpose of infection, rotavirus suppress MHC I expression *in vivo*, which might limit T cell-mediated killing [47]. However, rare literatures were reported the role of MHC I in the resistance to intracellular bacteria. In this study, we found that when rPoMHC Iα was added to the cell cultures of HKLs, it significantly enhanced cellular resistance against intracellular bacterial survival. RT-qPCR analysis showed that rPoMHC Iα induced the expression of multiple immune related factors. These results indicated PoMHC Iα activated $CD8^+$ T cells. *in vivo* experiment showed that following intracellular bacteria *E. tarda* infection, *PoMHC Iα* overexpressing flounder exhibited significantly lower bacterial amounts compared to control fish, and the mortality of *PoMHC Iα* overexpressing fish was significantly lower than those in the control fish. Consistent with the *in vivo* results, in vitro experiments showed that knockdown of *PoMHC Iα* attenuated the ability of host cell to clear pathogen. These results indicated that PoMHC Iα have remarkable effect on host defense against *E. tarda* infection. Recent research showed that MHC I appeared to play a critical role in host defense against intracellular bacterial pathogen *Coxiella burnetii* infection [50]. These experiments highlighted the importance of PoMHC Iα products for the control *E. tarda* infection.

In conclusion, we reported for the first time the immunological function of teleost MHC I homologue, PoMHC Iα, from *P. olivaceus*. The expressions of *PoMHC Iα* was significantly upregulated by extracellular and intracellular bacterial pathogen and viral pathogen. Expression pattern of *PoMHC Iα* appeared pathogen-dependent and time-

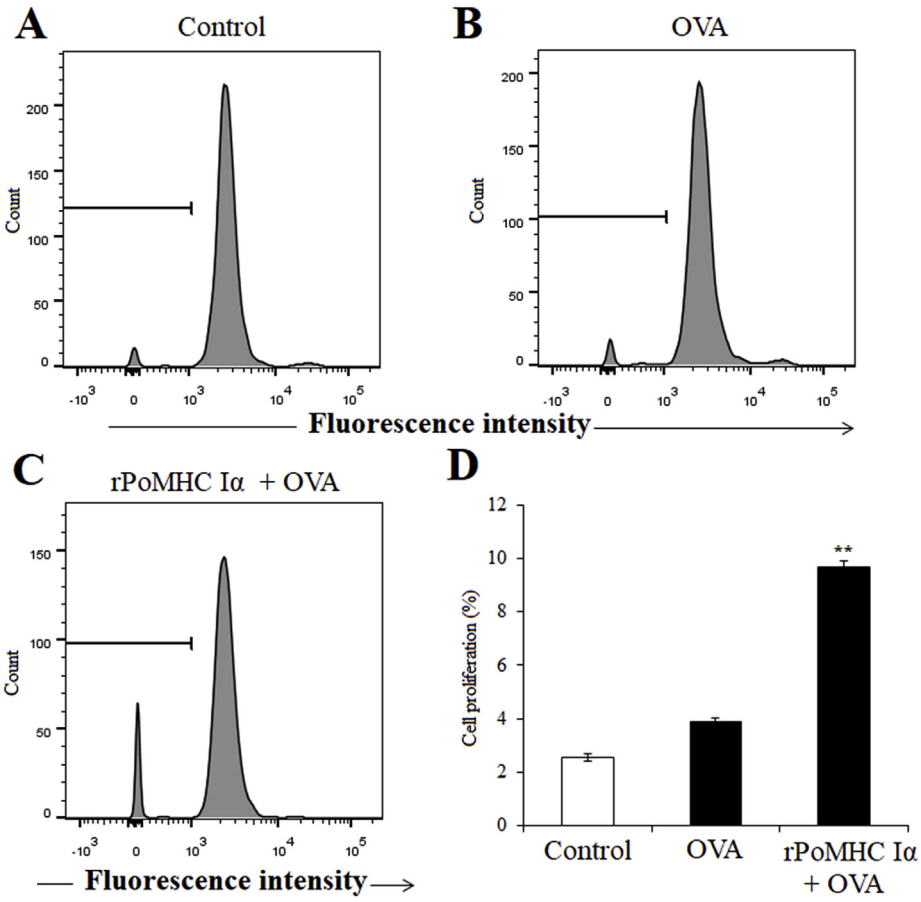


**Fig. 9. Effect of rPoMHC Iα on cells proliferation.** PBLs were stained by CFSE and stimulated with OVA or with OVA and rPoMHCI α. After incubated 24 h, cells proliferation and fluorescence intensity were analyzed by FCM. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. **, $P < 0.01$.

dependent. rPoMHC Iα enhanced cellular resistance against intracellular pathogen infection. The knockdown of *PoMHC Iα* attenuated the ability of host cells to eliminate intracellular pathogen *E. tarda*, which was sustained by the *in vivo* results that overexpression of *PoMHC Iα* promoted the host defense against invading *E. tarda*. The antigen presentation function of PoMHC Iα also was confirmed by antigen uptake assay. These findings add new insights into the biological function of teleost MHC Iα and emphasize the importance of MHC I gene products for the control of *E. tarda* infection.

## Acknowledgments

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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.2019.09.005.

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