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RESEARCH PAPER

Cloning, Expression, and Functional Analyses of MHC Class II A in Ayu, *Plecoglossus altivelis*

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Abstract

The MHC (major histocompatibility complex) class II family plays a major role in vertebrate immune systems and is normally found only on antigen-presenting cells. In this study, we identified the gene encoding the α (PaMHCIIα) chain of MHC class II molecules in ayu (*Plecoglossus altivelis*). Phylogenetic analysis showed that PaMHCIIα was tightly grouped with MHC class IIα in rainbow smelt (*Osmerus mordax*) and salmon. PaMHCIIα transcripts were detected in all tested tissues, with high levels in the spleen, head kidney, and gill; expression increased significantly upon *Vibrio anguillarum* infection. PaMHCIIα mRNA expression was also up-regulated in monocytes/macrophages (MO/MΦ) after *in vitro* stimulation with *V. anguillarum*. We prepared antibodies for detecting N-glycosylation of PaMHCIIα or neutralizing PaMHCIIα on ayu MO/MΦ. Western blot analysis showed that native PaMHCIIα was indeed N-glycosylated. Moreover, PaMHCIIα neutralization not only significantly inhibited ayu MO/MΦ phagocytosis, but also reduced the ability of *V. anguillarum* to induce MO/MΦ-cytokine expression. Overall, these findings reveal that the important role of PaMHCIIα against bacterial challenge, and it likely contributes to pathogen responsiveness in ayu MO/MΦ.

Keywords: Ayu, MHC class II α,vibrio anguillarum,gene expression, monocyte/macrophage functions.

Introduction

The highly polymorphic major histocompatibility complex (MHC) plays a critical role in innate and acquired immunity of vertebrates. Sequence, molecular structure, and functional analyses have identified MHC class I and class II in vertebrates. The MHC class I protein presents processed peptides from endogenously produced antigens to CD8-positive T cells (Di Pucchio*et al*., 2008), while the MHC class II protein presents exogenous antigens from extracellular pathogens to CD4-positive T cells (Schmid, Pypaert, & Münz, 2007). MHC class II proteins are non-covalently bound heterodimers of polypeptide chains, α and β chains (α and β genes). In mammals, MHC class II is constitutively expressed on antigen-presenting cells, including B cells, macrophages, monocytes, and dendritic cells (Ting & Trowsdale, 2002). They can be expressed on other cell types after stimulation with cytokines like interleukins (ILs) and interferons (IFNs) (Ting & Trowsdale, 2002).

MHC class II α sequences have been cloned and identified in several teleosts, including gilthead seabream (*Sparus aurata*) (Cuesta, Esteban, & Meseguer, 2006), large yellow croaker (*Larimichthys crocea*) (Yu, Ao, & Chen, 2010), Miiuy croaker (*Miichthys miiuy*) (Xu, Sun, Shi, Cheng, & Wang, 2011), Nile tilapia (*Oreochromis niloticus*) (Pang *et al*., 2013), and blunt snout bream (*Megalobrama amblycephala*) (Luo *et al*., 2014). Pathogen infection or immune stimulation can dramatically increase mRNA expression of MHC class II α (Cuesta, Esteban, & Meseguer, 2006; Yu, Ao, & Chen, 2010; Luo *et al*., 2014). For example, incubation with heat-killed *Saccharomyces cerevisiae* or *Vibrio anguillarum* significantly up-regulated transcription in head-kidney leucocytes of gilthead seabream (Cuesta, Esteban, & Meseguer, 2006). Stimulation with the trivalent bacterial vaccine or polyinosinic polycytidylic acid (polyI:C) elevated expression in the intestine, kidney, and spleen of large yellow croaker (Yu,Ao, & Chen, 2010). Furthermore, expression increased in the gill, kidney, intestine, and liver of blunt snout bream challenged with *Aeromonas hydrophila* (Luo *et al*., 2014).

Antibodies against MHC class II α are available to map MHC classII distribution and glycosylation in cells/tissues of several fish species (Nath, Kales, Fujiki, & Dixon, 2006; Scharsack, Kalbe, & Schaschl,

2007). As a result, MHC class II α proteins have been detected in channel fish (*Ictalurus punctatus*) clonal T-cell line 28S (Thankappan, Fuller, Godwin, Kearse, & McConnell, 2006), rainbow trout (*Oncorhynchus mykiss*) peripheral bloodleucocytes (PBLs) (van Lierop *et al*., 1998), along with other tissues (e.g., head kidney, gill, spleen, hindgut, liver, and muscle) (Nath, Kales, Fujiki, & Dixon, 2006; Scharsack, Kalbe, & Schaschl, 2007). The protein class's native form is N-glycosylated (van Lierop *et al*., 1998; Nath, Kales, Fujiki, & Dixon, 2006).

Ayu (*Plecoglossus altivelis*) is aneconomically important teleost widely cultured in East Asia (Japan, China, and Korea). Recently, high rearing densities among farm-raised ayu have led to severe *V. anguillarum*infection, seriously constraining ayu culture in China (Li, Chen, Shi, & Li, 2009). Given the important role of MHC class II in teleost immune systems, disease control and prevention would greatly benefit from understanding ayu MHC class II α (PaMHCIIα) function and mechanisms of action. Thus, in this study, we decided to identify the cDNA sequence of the PaMHCIIα gene, along with its mRNA expression profiles and link to *V. anguillarum* infection, and investigate the effects of recombinant PaMHCIIα on the phagocytosis of ayu monocytes/macrophages (MO/MΦ), along with cytokine mRNA expression.

Materials and Methods

Fish Rearing, Sampling and Pathogen Challenge

Ayu(each 40–50 g) were obtained from a commercial farm in Fuxi, Ninghai County, Ningbo City, China. The fish were acclimated in 100 L tanks with a recirculating system at 20 ± 0.5°C with filtered freshwater for two weeks. Only fish with no visible pathological signs were used for experiments. All animal care and experimental procedures were approved by the Committee on Animal Care and Use and the Ethics Committee for Animal Experiments at Ningbo University.

The *V. anguillarum* challenge followed procedures from Chen *et al*. (2014). Infected ayu were injected intraperitoneally with 100 μL bacterial suspension (1.2×10^5 colony-forming units [CFU]/mL), while control fish were injected with PBS and kept in separate tanks. Individuals from both groups of fish were killed at 4, 8, 12, and 24 h post-infection (hpi). Liver, spleen, kidney, head kidney, gill, intestine, and PBLs were collected, immediately snap-frozen in liquid nitrogen, and preserved at -80°C until RNA extraction.

Cell Preparations

Existing procedures were followed to purify PBLs from blood samples (Lam *et al*., 2011). Head-kidney-derived MO/MΦ cells were also isolated and cultured as previously described (Zhang, Shi, & Chen, 2015). Briefly, the head-kidney leukocyte-enriched fraction was obtained with Ficoll-Hypaque PREMIUM (1.077 g/mL) (GE Healthcare, New Jersey, USA), following manufacturer protocol. Non-adherent cells were washed off, and adherent cells were kept in a complete medium (RPMI 1640, 4% ayu serum, 6% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) at 24°C with 5% CO_2.

Molecular Cloning and Characterization of Pamhciiα Cdna

Transcriptome data of head-kidney-derived MO/MΦ were used to obtain PaMHCIIα cDNA that was then verified with PCR, TA-cloning, and sequencing. Multiple alignment and phylogenetic reconstruction were performed in ClustalW (http://clustalw.ddbj.nig.ac.jp/) and MEGA 5.0 (Tamura *et al*., 2011), respectively. PaMHCIIα domain architecture and the potential N-glycosylation site were predicted in SMART (http://smart.embl-heidelberg.de/) (Letunic, Doerks, & Bork, 2015) and NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

In vitro Stimulation of AyuMO/MΦ with *V. Anguillarum*

Stimulation of ayu MO/MΦ with *V. anguillarum* was performed as previously described (Rong, Lu, & Chen, 2016). Cells were switched to antibiotic-free medium and incubated for another 12 h before infection with live *V. anguillarum* at a multiplicity of infection (MOI) of 2. The control group was treated with PBS. Treated and untreated cells were harvested at 4, 8, 12, and 24 h post-treatment, then preserved at -80°C before use.

Real-Time Quantitative PCR (qPCR)

Total RNA extraction, DNase I digestion, first-strand cDNA synthesis, and qPCR were performed as previously reported (Yong, Lu, & Chen, 2016). The qPCR was performed using SYBR premix Ex Taq (Perfect Real Time; TaKaRa, Dalian, China) in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA). Table 1 shows the primers for the analysis.The PaMHCIIα mRNA expression was normalized to 18S rRNA (the internal control) using the $2^{-\Delta\Delta CT}$ method.

Prokaryotic Expression and Antibody Preparation

The primer pair PaMHCIIαex(+) and PaMHCIIαex(-) was designed to amplify the extracellular region (residues 19–195) of PaMHCIIα (PaMHCIIαex) (Table 1). To construct the recombinant plasmid (pET28a-PaMHCIIαex),

Table 1. Oligonucleotide primers used in this study

Gene	Primer	Accession number	Nucleotide sequence (5′→3′)	Length (bp)
MHCIIα	PaMHCIIα(+)	KX118042	TGACACGGATATGGGATGTG	110
	PaMHCIIα(-)		GAAGAATGTTCCAGCAGCCA	
MHCIIα	PaMHCIIαex(+)[a]	KX118042	*G*GAATTCCTAATTACACATGAGGATATCA	531
	PaMHCIIαex(-)[b]		*C*CTCGAGCCATATCCGTGTCAGCGGCT	
TNF-α	PaTNFα(+)	JP740414	ACATGGGAGCTGTGTTCCTC	115
	PaTNFα(-)		GCAAACACACCGAAAAAGGT	
IL-1β	PaIL-1β(+)	HF543937	TACCGGTTGGTACATCAGCA	104
	PaIL-1β(-)		TGACGGTAAAGTTGGTGCAA	
IL-10	PaIL-10(+)	JP758157	TGCTGGTGGTGCTGTTTATGTGT	73
	PaIL-10(-)		AAGGAGCAGCAGCGGTCAGAA	
TGF-β	PaTGF-β(+)	JP742920	CTGGAATGCCGAGAACAAAT	101
	PaTGF-β(-)		GATCCAGAACCTGAGGGACA	
18S rRNA	18S rRNA(+)	FN646593	GACACGGAAAGGATTGACAG	119
	18S rRNA(-)		CGGAGTCTCGTTCGTTAT	

[a] and [b]: The underlined sections represent *Eco*RI (in PaMHCIIαex(+)) and *Xho*I (in PaMHCIIαex(-)) restriction sites, respectively

amplicons were digested with *Eco*RI and *Xho*I, then inserted into the multiple cloning site of vector pET28a. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3). After IPTG induction, the recombinant PaMHCIIαex protein (rPaMHCIIαex) with His-tag was purified using a nickel-nitrilotriacetic acid column (Ni-NTA) (Qiagen, Hilden, Germany), following manufacturer protocol. The presence of rPaMHCIIαex was confirmed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, rPaMHCIIαex was inoculated into mice to produce antiserum. Antibody production and detection was performed as previously described (Zhang, Shi, & Chen, 2015).

Deglycosylation and Western Blot Analysis

To determine whether native PaMHCIIα is N-glycosylated, denatured ayu MO/MΦ were treated with PNGase F (New England Biolabs, Beverly, USA) and endoglycosidase H (New England Biolabs) as previously described (Nath, Kales, Fujiki, & Dixon, 2006). In brief, ayu MO/MΦ were lysed in RIPA buffer (Beyotime, Shanghai, China), and 500 μg of lysate was boiled for 10 min in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). After the addition of endoglycosidase H (2500 units) and sodium citrate buffer (pH 7.5), samples were left overnight at 37°C. A second overnight digestion was performed at 37°C with 20 U of PNGase F in sodium phosphate buffer (pH 7.5) and 1% NP-40. The western blot analysis, SDS-PAGE, membrane transfer, antibody incubation, and electrochemiluminescence reaction were performed as described by Li *et al*. (2015).

Immunofluorescence Assays

Existing methods were followed for immunofluorescence assays (Ao, Ling, & Yu, 2008). Briefly, head-kidney cells and PBLs were fixed in 4% paraformaldehyde for 30 min, air-dried, then soaked in PBS. Cells were blocked with 5% BSA in PBS before incubation with a polyclonal mouse antibody to PaMHCIIαex solution (1:50 dilution) at 4°C overnight. Cells were visualized with a laser confocal microscope IX81-FV1000 (Olympus, Tokyo, Japan) using DyLight™ 488-TFP ester-conjugated goat-antimouse IgG (1:200 in PBS). Cell nuclei were stained with DAPI (10 μg/mL, Sigma-Aldrich, Shanghai, China).

Phagocytosis Assay

E. coli DH5α cells at the logarithmic growth phase were labeled with fluorescein isothiocyanate (FITC; Sigma, St. Louis, USA) (FITC-DH5α) for the phagocytosis assay. Ayu MO/MΦ grown in six-well plates (2×10^6 cells/well) were blocked with 200 μg/mL anti-PaMHCIIαex IgG for 30 min. FITC-DH5α was added at an MOI of 20, and the control was PBS + mouse isotype IgG. After extensive washing with sterile PBS to remove extracellular particles, ayu MO/MΦ was further incubated for 30 min. Trypan blue (0.4%) was used to quench extracellular fluorescence and a Gallios flow cytometer(Beckman Coulter, Miami, USA) was used to examine MO/MΦ bacterial uptake.

MO/MΦ Cytokine mRNA Expression Assay

For PaMHCIIα neutralization, ayu MO/MΦ were pre-incubated with anti-PaMHCIIαex IgG (200 μg/mL) or mouse isotype IgG (200 μg/mL) for 30 min. Next, *V. anguillarum* was added at an MOI of 2, and incubated with the cells for 8 h, with PBS as the control. At 8 hpi, cells were collected to measure the transcript changes of TNF-α, IL-1β, IL-10, and TGF-β.

Statistical Analysis

Data are presented as mean ± SD and analyzed with one-way analysis of variance (ANOVA) in SPSS version 13.0 (SPSS Inc., Chicago, USA). Statistical significance was set at $P<0.05$.

Results

Molecular Characterization of PaMHCIIα cDNA

The size of PaMHCIIα cDNA (GenBank no. KX118042) was 1358 bp, including a 5′- untranslated region (UTR) of 239 bp and 3′-UTR of 411 bp. The 708-bp open reading frame of PaMHCIIα encoded a polypeptide of 235 amino acids (aa), with a putative molecular weight (MW) of 25.61 kDa and a theoretical isoelectric point (pI) of 4.78. The PaMHCIIα sequence had an 18-aa signal pepitde (SP), two extracellular domains (α1: residues 19–111; α2: residues 112–195), and a conserved connecting peptide/transmembrane/cytoplasmic (CP/TM/CYT) domain (residues 196–235) (Figure 1), all typical characteristics of the MHC class II. The PaMHCIIαsequence also contained conserved cysteine residues that form the intra-domain disulfide bridges (α1: residues 30 and 83; α2: residues 124 and 180), as well as the conserved site for potential N-glycosylation in the α1 domain (residues 144–146) (Figure 1). Finally, we observed the conserved GxxxGxxGxxxG motif (where x is any hydrophobic residue other than Gly) in the TM region (Figure 1).

Sequence comparisons revealed that PaMHCIIα shared the highest aa identity (77.5%) with rainbow smelt (*Osmerus mordax*) MHC class II α. Additionally, phylogenetic analysis revealed that vertebrate MHC class II α sequences fall into two major clusters: the mammalian-reptile-bird group and the fish group. PaMHCIIα is most closely related to rainbow smelt MHC class II α, but more distant from the sequences in Atlantic salmon (*Salmo salar*), Arctic charr (*Salvelinus alpinus*), and rainbow trout (Figure 2), reflecting currently accepted relationships.

PaMHCIIα Gene Expression in *V. anguillarum*-Infected Ayu

PaMHCIIα mRNA expression was detected in all tested tissues, PBLs, and MO/MΦ of healthy ayu; expression levels were higher in the spleen, intestine, and gill (Figure 3A). Following *V. anguillarum* challenge, PaMHCIIα mRNA significantly increased in the liver (all time points; Figure 3B), spleen (12 hpi; Figure 3C), kidney (12 hpi; Figure 3D), head kidney (4, 8, and 12 hpi; Figure 3E), intestine (all time points; Figure 3F), gill (8, 12, and 24 hpi, Figure 3G), and PBLs (4, 8, and 12 hpi, Figure 3H). Post-challenge PaMHCIIα expression was also clearly up-regulated in ayu MO/MΦ (at 8, 12, and 24 hpi) (Figure 3I).

Production of Anti-PaMHCIIαexAntibody and Immunofluorescence Analysis of PaMHCIIα

PaMHCIIαex was overexpressed in BL21 (DE3) cells transformed with the pET28a-PaMHCIIαex plasmid (Figure 4A, lanes 1–2). The results of SDS-PAGE indicated that rPaMHCIIαex (extracted from inclusion bodies) was highly pure after treatment using molecular sieve filtration (Figure 4A, lane 3). The MW of rPaMHCIIαex was 23.70 kDa, matching with the expected size (19.86 kDa PaMHCIIαex+ 3.84 kDa His-tag).

The PaMHCIIαex polyclonal antibody detected one protein band at about 24 kDa in the recombinant protein (Figure 4B, lane 1). In ayu MO/MΦ, the PaMHCIIα protein had a MW of approximately 34 kDa (Figure 4B, lane 2). Incubation of denatured (boiling in SDS) MO/MΦ lysates with endoglycosidase H and PNGase F altered the electrophoretic mobility pattern of the protein band from 34 to 31 kDa (Figure 4B, lane 3).

Immunofluorescence analysis showed that PaMHCIIα proteins were widely expressed in head-kidney cells and PBLs cultured with the anti-PaMHCIIαex antibody (Figure 5).

PaMHCIIα Effect on MO/MΦ Phagocytosis and Cytokine mRNA

The ability of MO/MΦ to phagocytize *E. coli*-FITC changed significantly after PaMHCIIα neutralization by anti-PaMHCIIαex IgG (Figure 6A and 6B). After PaMHCIIα neutralization, MO/MΦ phagocytosis of *E. coli*-FITC decreased by 77.9% (Figure 6B).

After *V. anguillarum* infection, anti-PaMHCIIαex IgG-treated MO/MΦ experienced a significant decrease in the mRNA expression of TNF-α, IL-1β, IL-10, and TGF-β compared with the isotype IgG-treated MO/MΦ (Figure 7A-D). Post-infection cytokine mRNA expression in both types of treated MO/MΦ was also significantly higher than that in untreated MO/MΦ (Figure 7A-D).

Discussion

In the present study, we cloned the ayu MHC class II α gene (PaMHCIIα) and characterized its structure. The deduced peptide contained typical structural features of the class—including the SP, CP/TM/CYT, and extracellular (α1/α2) domains—consistent with other species (Cuesta, Esteban, & Meseguer, 2006; Xu, Sun, Shi, Cheng, & Wang, 2011; Luo *et al*., 2014; Wang, Tan, & Cai, 2015). Moreover, the TM region of PaMHCIIα contained the GxxxGxxGxxxG motif, shared across fishes and mammals (Cosson & Bonifacino, 1992; Xu, Sun, Shi, Cheng, & Wang, 2011; Jiang, Li, Zhang, & Wang, 2013; Luo *et al*., 2014; Wang, Tan, & Cai, 2015). PaMHCIIα has only one N-linked glycosylation site,

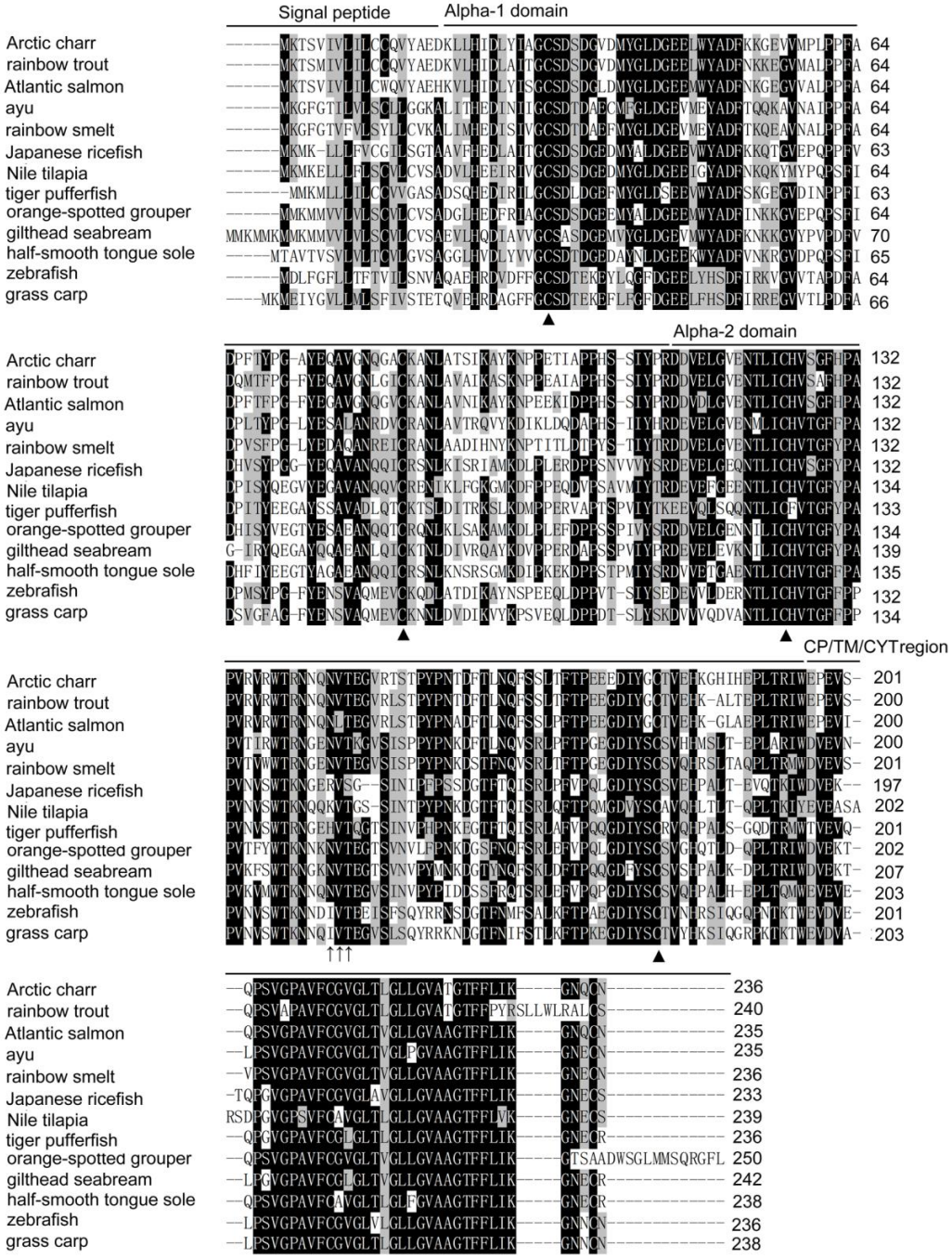

Figure 1. Multiple alignment of the amino acid sequences of PaMHCIIα and other closely related fish MHC class II α molecules. Threshold for shading is >60%, similar residues are marked with gray shading, identical residues are marked with black shading, and alignment gaps are marked as "–". The conserved cysteine residues forming disulfide bridges are marked with "▲" and N-glycosylation site is marked with "↑". Accession numbers of sequences used are ayu (*Plecoglossus altivelis*),KX118042;rainbow smelt (*Osmerus mordax*), ACO09715; Atlantic salmon (*Salmo salar*), AGH92604; Japanese ricefish (*Oryzias latipes*), XP_004084698; smooth tongue sole (*Cynoglossus semilaevis*), NP_001281129; Nile tilapia (*Oreochromis niloticus*), AEO44574; orange-spotted grouper (*Epinephelus coioides*), AEA39745; seabream (*Sparus aurata*), AAY42849; Arctic charr (*Salvelinus alpinus*), ACI05079; rainbow trout (*Oncorhynchus mykiss*), CAB96452; pufferfish (*Takifugu rubripes*), BAH30162; zebrafish (*Danio rerio*), AAA16369; and grass carp (*Ctenopharyngodon idella*), ABW37740.

consistent with most teleosts (Nath, Kales, Fujiki, & Dixon,2006; Cuesta, Esteban, & Meseguer, 2006; Yu, Ao, & Chen, 2010; Jiang, Li, Zhang, & Wang, 2013; Luo *et al*., 2014) except zebrafish (Sültmann, Mayer, Figueroa, O'hUigin, & Klein, 1993), channel catfish (Thankappan, Fuller, Godwin, Kearse, & McConnell, 2006), andstickleback (*Gasterosteus aculeatus*) (Scharsack, Kalbe, & Schaschl,2007). In contrast, human and mouse MHC class II α proteins have two N-linked glycosylation sites (Nag *et al*., 1994;

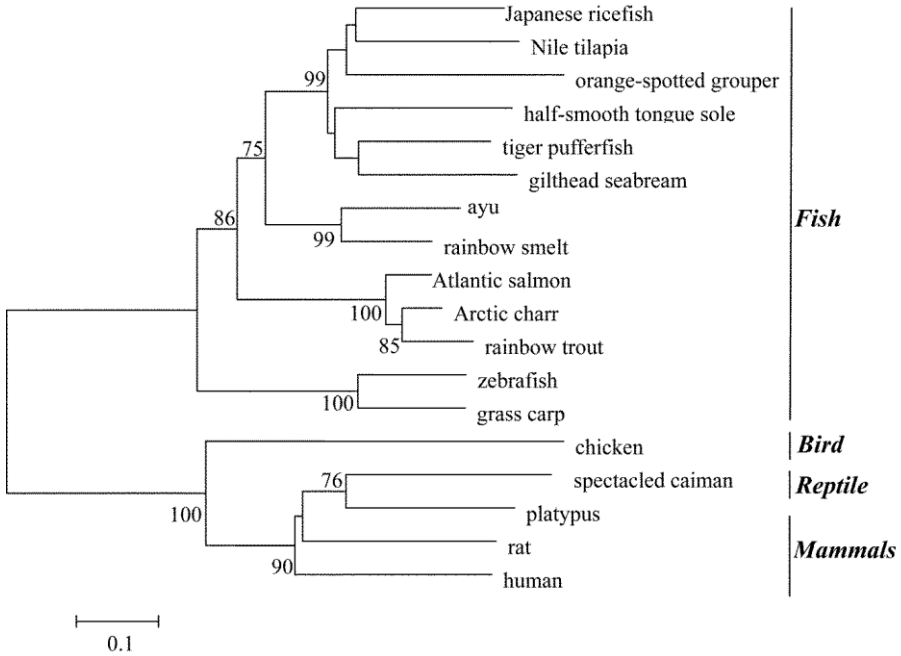

Figure 2. Neighbor-joining phylogenetic analysis (in MEGA5.0) of amino acid sequences from the deduced MHC class II α molecules. Numbers at the nodes indicate percentage of trees (>60%) that included the grouping (after bootstrapping for 1000 replicates). The scale bars show the number of substitutions per base. Accession numbers of fish MHC class II αsequences used are listed in the legend offigure 1, and accession numbers of other species MHC class II α sequences used are chicken (*Gallus gallus*), AAY40299; African clawed frog (*Xenopus laevis*), NP_001083579; rat (*Rattus norvegicus*), AAR87772; human (*Homo sapiens*), AAP80750; platypus (*Ornithorhynchus anatinus*), ABU86899; and spectacled caiman (*Caiman crocodilus*), AAF99282.

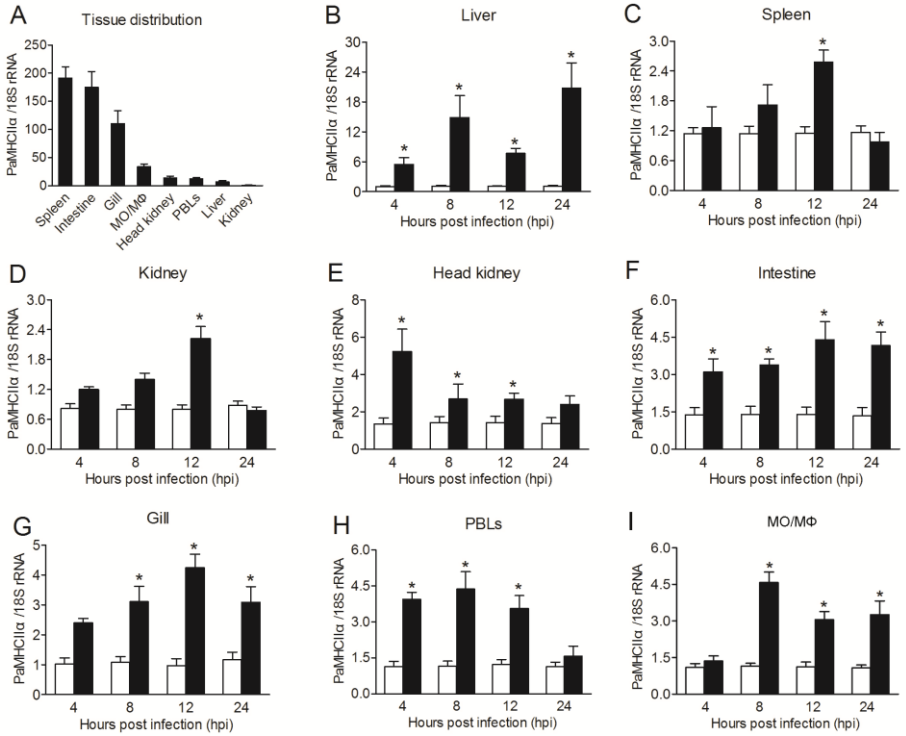

Figure 3. Expression analysis of PaMHCIIα in healthy fish or fish challenged with *V. anguillarum*. (A) PaMHCIIα mRNA expression in healthy ayu tissues. (B-H) PaMHCIIα mRNA expression in tissues or PBLs (peripheral blood leucocytes) of ayu challenged with *V. anguillarum*. (I) PaMHCIIα mRNA expression in ayu head-kidney-derived MO/MΦ infected with *V. anguillarum*. Samples were collected at different time points after *V. anguillarum* challenge (x-axis). PaMHCIIα transcript levels were normalized to the ayu 18S rRNA gene. Data are expressed as mean ± SD (n = 4). *: $P < 0.05$.

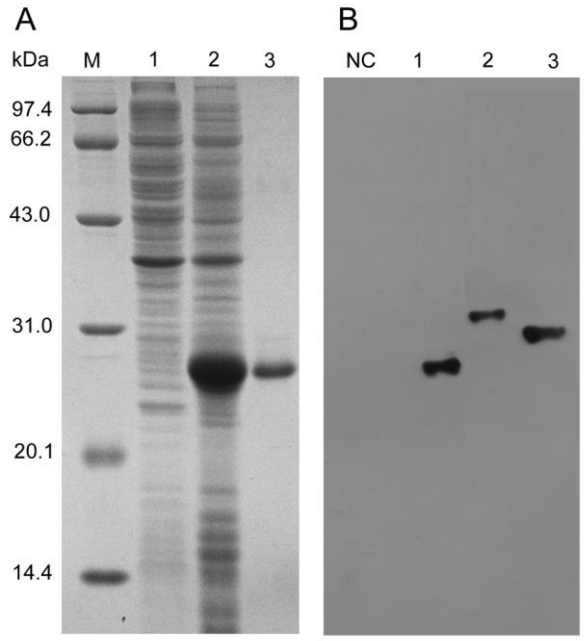

Figure 4. Prokaryotic expression and western blot analysis of PaMHCIIα. (A) SDS-PAGE analysis of recombinant extracellular PaMHCIIα peptides. Lane M: protein marker; 1: before IPTG induction; 2: after IPTG induction; 3: purified recombinant protein. (B) Western blot analysis of recombinant extracellular PaMHCIIα and native PaMHCIIα in MO/MΦ. NC: negative control; 1: purified recombinant protein; 2: total protein extracted from ayu MO/MΦ; 3: total protein extracted from ayu MO/MΦ, digested with endoglycosidase H and PNGase F.

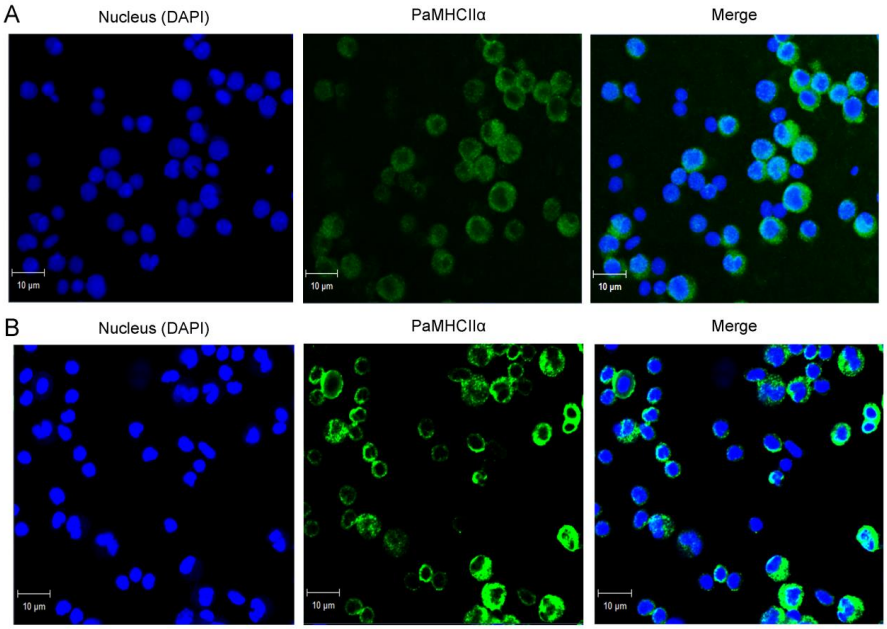

Figure 5. Immunofluorescence assays of PaMHCIIα in ayu head-kidney cells and PBLs. Isotype IgG was used as a negative control. Indirect immunofluorescence antibody tests were used for the detection of PaMHCIIα-positive cells in ayu head-kidney cells (A) and PBLs (B). Green and blue represent the distribution of PaMHCIIα molecules and cell nuclei, respectively.

Ishikawa, Kowal, Cole, Thomson, & Diamond, 1995). Phylogenetic analysis revealed that the MHC class II α of ayu and rainbow smelt share the highest aa identity (77.5%). Overall, the high degree of conservation in MHC class II α structures suggests that the protein group probably plays vital roles in vertebrates.

We observed that PaMHCIIα mRNA was expressed in all tested tissues/cells, and had particularly high expression in the spleen, intestine, and gill, consistent with findings in other teleosts. For example, in large yellow croaker, MHC class II α

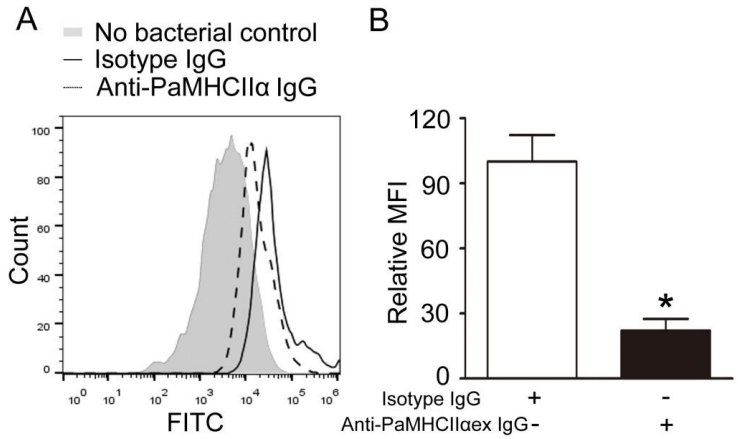

Figure 6. Effect of PaMHCIIα neutralization on ayu MO/MΦ phagocytosis. Anti-PaMHCIIαex IgG blocked PaMHCIIα on MO/MΦ, while isotype IgG was the control. (A) Bacterial uptake was analyzed using a Gallios flow cytometer. (B) The relative mean fluorescence intensity (MFI) of anti-PaMHCIIαex IgG- and isotype IgG-treated groups were expressed in fold change. The isotype control was assigned a value of 100. Data are expressed as the mean ± SD (n = 3). *P< 0.05.

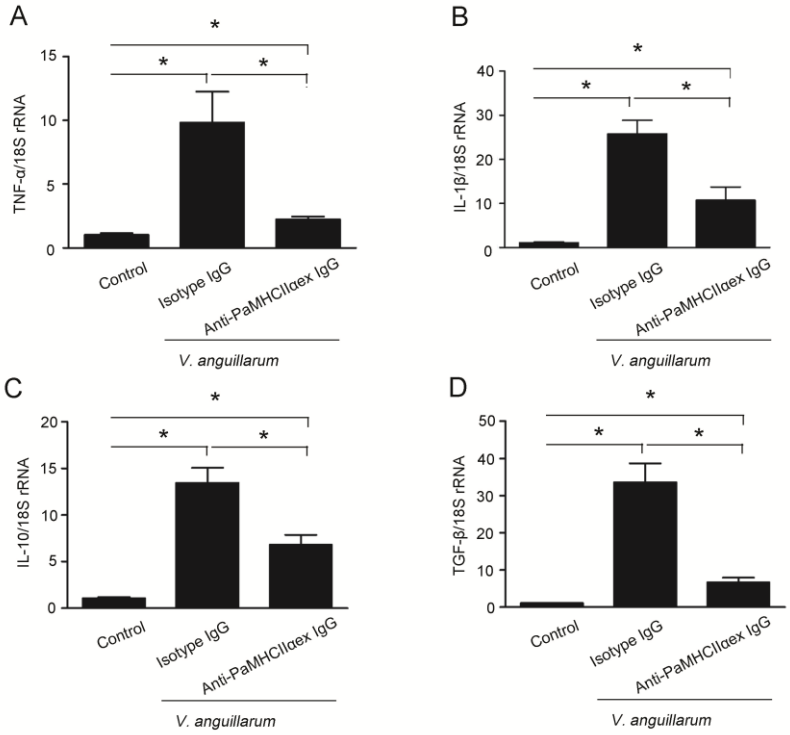

Figure 7. Effect of PaMHCIIα neutralization on the mRNA expression of TNF-α, IL-1β, IL-10, and TGF-β in *Vibrio anguillarum*-challenged ayu MO/MΦ. Real-time quantitative PCR was used to determine mRNA expression of TNF-α (A), IL-1β (B), IL-10 (C), and TFG-β (D). MO/MΦ cells were incubated with anti-PaMHCIIαex IgG for 30 min to block PaMHCIIα. Isotype IgG-treated groups were the control. *V. anguillarum* was added to the cell culture media at MOI = 2, followed by incubation for another 8 h. Data are expressed as the mean ± SD (n = 3). *P<0.05.

mRNA was highly expressed in kidney, intestine, gills, and spleen (Yu, Ao, & Chen, 2010). Similarly, in stone flounder and blunt snout bream, MHC class II α mRNA expression was highest in the gills, followed by intestines and spleen (Jiang, Li, Zhang, & Wang, 2013; Luo *et al*., 2014). Finally, in gilthead seabream, MHC class II α mRNA was expressed in head-kidney leucocyte subpopulations, including acidophilic granulocytes, lymphocytes, and MO/MΦ (Cuesta, Esteban, & Meseguer,2006). Overall, existing data suggest that PaMHCIIα protein exists widely in PBLs and head-kidney cells. Furthermore, our data combined with previous work showed a rapid response of MHC class II α to pathogen infection,

with clear up-regulation of transcripts during early inflammation (Cuesta, Esteban, & Meseguer, 2006; Yu, Ao, & Chen, 2010; Luo *et al*., 2014).

In the present study, western blots revealed that PaMHCIIα in ayu MO/MΦ had higher MW than expected from the mature PaMHCIIα aa sequence (23.82 kDa). However, after digestion with endoglycosidase H and PNGase F, PaMHCIIα in ayu MO/MΦ exhibited the expected, lower MW. In accordance with our study, the MW of native MHC class II α from rainbow trout gills and PBLs was approximately 32 kDa, higher than the aa-derived MW; after deglycosylation, MW was around 29 kDa (van Lierop *et al*., 1998; Nath, Kales, Fujiki, & Dixon, 2006).These results demonstrated that PaMHCIIα was definitely N-glycosylated and constituted about 10% of the glycoprotein's total mass. Notably, in channel catfish and stickleback, the molecular mass of native MHC class II α matched completely with the MW calculated from the aa sequence, confirming that glycosylation sites are absent (Thankappan, Fuller, Godwin, Kearse, & McConnell, 2006; Scharsack, Kalbe, & Schaschl, 2007).

Prior to our study, we knew little about the role of MHC class II in MO/MΦ of the fish immune system. Here, we demonstrated that PaMHCIIα neutralization significantly attenuated ayu MO/MΦ phagocytosis and effectively eliminated TNF-α, IL-1β, IL-10, and TGF-β expression in ayu MO/MΦ upon *V. anguillarum* infection. These outcomes indicate that PaMHCIIα likely contributes to pathogen responsiveness in ayu MO/MΦ. This general function accords with our understanding of the better-studied mammalian MHC class II proteins, involved in regulating immune cell function, including cell adhesion, cytokine production, apoptosis, proliferation, and B lymphocyte differentiation (Piani *et al*., 2000; Liu*et al*., 2011). For example, in human peripheral blood monocytes or monocytic cells, MHC class II deficiencies decreased the production of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines (TNF-α, IL-1, or IL-8) (Piani *et al*., 2000). Our study provides promising information on the role of MHC class II α proteins in ayu immune defense against infection, but future studies should investigate the detailed mechanisms underlying the link between PaMHCIIα and ayu MO/MΦ function.

In summary, we have identified a novel MHC class II α protein from ayu. PaMHCIIα transcripts were significantly up-regulated in various tissues and cells upon bacterial infection. We demonstrate that the PaMHCIIα neutralization can inhibit cytokine mRNA expression and ayu MO/MΦ phagocytosis. We believe that the present study will contribute to a better understanding of fish MHC class II function.

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