

# Molecular Cloning of The Major Histocompatibility Complex IIB and its Function Identification in Ayu (*Plecoglossus altivelis*) on *Vibrio anguillarum* Infection

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**Abstract** The major histocompatibility complex class II (MHC II) plays pivotal roles in the immune system of vertebrates. We obtained the gene (*PaMHCIIB*) encoding the  $\beta$  chain of MHC II molecule in ayu (*Plecoglossus altivelis*), with *de novo* transcriptome sequencing of ayu monocytes/macrophages (MO/M $\Phi$ ). The full-length cDNA sequence of the gene was 920 nucleotides, containing a large open reading frame that encoded 251 amino acids, and the molecular mass was deduced to be 28.23 kD. Amino acid sequence analysis showed that PaMHCIIB possessed the typical signatures of the MHC IIB, including the signal peptide, two extracellular domains, and one connecting peptide/transmembrane/cytoplasmic (CP/TM/CYT) domain. PaMHCIIB shared the highest amino acid sequence identity (65.04%) with that of Arctic charr (*Salvelinus alpinus*). Phylogenetic analysis showed that PaMHCIIB was most closely related to the MHC IIB of Arctic charr. Quantitative real-time PCR (qPCR) analysis showed that the *PaMHCIIB* transcript was mainly expressed in the gill, intestine, and spleen. After infection by *Vibrio anguillarum*, *PaMHCIIB* mRNA in the liver was significantly upregulated at 12 hours post infection (hpi), reaching a peak value of 3.18-fold of at 24 hpi ( $P < 0.05$ ); *PaMHCIIB* mRNA in the spleen, head kidney, intestine, and gill were all significantly upregulated at 4 hpi, reaching the peak values of 85.18-, 2.06-, 4.21-, and 6.81-fold, at 4, 8, 24, and 12 hpi ( $P < 0.05$ ), respectively. The *PaMHCIIB* transcription increased in MO/M $\Phi$  after *in vitro* stimulation with *V. anguillarum*, reaching a peak value of 3.35-fold at 12 hpi ( $P < 0.05$ ). The extracellular domain of PaMHCIIB (PaMHCIIBex) was overexpressed, and the antibodies against PaMHCIIBex were prepared for detecting N-glycosylation of PaMHCIIB or for neutralizing PaMHCIIB in ayu MO/M $\Phi$ . Western blotting analysis showed that the native PaMHCIIB protein was definitely N-glycosylated in ayu MO/M $\Phi$ , and the PaMHCIIB protein level also increased in MO/M $\Phi$  after *in vitro* stimulation with *V. anguillarum*, reaching a peak value of 3.19-fold at 24 hpi ( $P < 0.05$ ). Furthermore, PaMHCIIB neutralization remarkably restrained the MO/M $\Phi$  phagocytosis in ayu (0.28-fold) ( $P < 0.05$ ), and the mRNA expression of *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-10*, and *TGF- $\beta$*  induced by *V. anguillarum* was also inhibited, reaching the minimum (0.23-, 0.41-, 0.51-, and 0.20-fold, respectively) at 8 hpi ( $P < 0.05$ ). Overall, these results demonstrate the crucial role of PaMHCIIB against bacterial challenge, and suggest its involvement in the host resistance to pathogen infection in ayu MO/M $\Phi$ .

**Key words** *Plecoglossus altivelis*; major histocompatibility complex class IIB; *Vibrio anguillarum*; gene expression; monocytes/macrophages

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# 香鱼主要组织相容性复合体 MHCIIB 的分子克隆及其在鳃弧菌感染下的功能鉴定

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**摘要** 主要组织相容性复合体(major histocompatibility complex class II, MHC II)在脊椎动物免疫反应中发挥重要作用。本研究从香鱼(*Plecoglossus altivelis*)单核/巨噬细胞(monocytes/macrophages, MO/MΦ)转录组中获得了 MHC II 基因 β 链(*PaMHCIIIB*) cDNA 序列。*PaMHCIIIB* 由 920 个核苷酸组成, 包含一个大的开放阅读框, 编码 251 个氨基酸, 预测分子质量为 28.23 kD。氨基酸序列分析表明, *PaMHCIIIB* 具有 MHC IIB 的典型特征, 主要包括信号肽、2 个胞外区和 1 个保守的 connecting peptide/transmembrane/cytoplasmic (CP/TM/CYT) 结构域, 与北极红点鲑(*Salvelinus alpinus*) MHC IIB 同源性最高, 为 65.04%; 系统发育分析表明, *PaMHCIIIB* 与北极红点鲑 MHC IIB 进化相关性最高。实时荧光定量 PCR (quantitative real-time PCR, qPCR) 结果显示, *PaMHCIIIB* mRNA 主要在香鱼鳃、肠和脾中表达; 鳃弧菌感染(*Vibrio anguillarum*)后香鱼肝中 *PaMHCIIIB* mRNA 在感染后 12 h (hours post infection, hpi) 时上调显著, 在 24 hpi 达到峰值, 为对照组的 3.18 倍, 脾、头肾、肠和鳃中 *PaMHCIIIB* mRNA 均在 4 hpi 时上调显著, 分别在 4、8、24 和 12 hpi 时达到峰值, 分别为对照组的 85.18、2.06、4.21 和 6.81 倍( $P < 0.05$ )。香鱼 MO/MΦ 经鳃弧菌感染后, *PaMHCIIIB* mRNA 的表达水平在 4 hpi 时上调显著, 在 12 hpi 时达到峰值, 为对照组的 3.35 倍( $P < 0.05$ )。原核表达了 *PaMHCIIIB* 胞外区并制备其抗体。Western 印迹分析结果表明, 香鱼 MO/MΦ 中 *PaMHCIIIB* 具有 N 糖基化修饰, 且鳃弧菌感染后其蛋白表达水平在 12 hpi 时显著增加, 在 24 hpi 时达到峰值, 为对照组的 3.19 倍( $P < 0.05$ )。抗体封闭 *PaMHCIIIB* 后, 香鱼 MO/MΦ 吞噬活性被抑制, 为对照组的 0.28 倍( $P < 0.05$ ), 而且鳃弧菌诱导的 4 个细胞因子 *TNF-α*、*IL-1β*、*IL-10* 和 *TGF-β* 表达均受到抑制, 均在 8 hpi 时被抑制最明显, 表达量分别为对照组的 0.23、0.41、0.51 和 0.20 倍( $P < 0.05$ )。本研究结果揭示 *PaMHCIIIB* 可能参与香鱼 MO/MΦ 抵抗病原菌感染的免疫防御。

**关键词** 香鱼; 主要组织相容性复合体; 鳃弧菌; 基因表达; 单核/巨噬细胞

**中图分类号** Q786; S917

The major histocompatibility complex class II (MHC II) plays a pivotal role in the immune system of vertebrates by presenting exogenous antigen peptides, derived from extracellular pathogens during infection to CD4-positive T cells. MHC II genes are constitutively expressed in specialized antigen-presenting cells (B cells, monocytes, macrophages and dendritic cells) and in thymic epithelial cells<sup>[1-2]</sup>. Their expression levels can be modulated after stimulation with cytokines like interleukin-10 (IL-10), tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ). And TNF-α can synergize with IFN-γ in inducing MHC II expression<sup>[1, 3-4]</sup>. In humans, the three isoforms of classical MHC II genes are HLA-DR, DP and DQ, each of which are non-covalently bound heterodimers of two polypeptide chains (α and β chains), and encoded by separate genes (A and B genes).

The MHC II genes have also been identified in

teleost. The fish MHC II genes also comprise of both the MHC IIA and IIB genes. MHC IIB genes have been characterized in several species, such as rainbow trout (*Oncorhynchus mykiss*)<sup>[5]</sup>, red sea bream (*Chrysophrys major*)<sup>[6]</sup>, large yellow croaker (*Larimichthys crocea*)<sup>[7]</sup>, Nile tilapia (*Oreochromis niloticus*)<sup>[8]</sup>, blunt snout bream (*Megalobrama amblycephala*)<sup>[9]</sup>, and European seabass (*Dicentrarchus labrax* L.)<sup>[10]</sup>. Studies have focused on the genomic organization, molecular polymorphism, evolution, glycosylation, and expression of these species<sup>[9, 11-14]</sup>. Expression studies have shown that the MHC IIB genes are expressed widely, with high levels detected in immune-related tissues, such as spleen, kidney, gill, and intestine<sup>[6, 9, 13, 15]</sup>. Moreover, immune stimulation or bacterial infection can dramatically increase the mRNA expression of MHC IIB<sup>[7, 9, 11, 16]</sup>. For instance, stimulation with the trivalent bacterial vaccine or polyinosinic: polycytidylic acid

(polyI:C) upregulated the expression of *MHC IIB* in intestine, kidney, and spleen of large yellow croaker<sup>[7]</sup>. Challenges with the extracellular pathogen *Aeromonas hydrophila* in the blunt snout bream significantly increased the expression of *MHC IIB* mRNA within 72 h in the gill, kidney, intestine, and liver<sup>[9]</sup>. However, challenging the red sea bream with *Vibrio anguillarum* downregulated the transcription of *MHC IIB* mRNA from 5 to 72 h after infection in the liver, spleen, head kidney, and intestine<sup>[6]</sup>.

Antibodies are used to detect MHC IIB distribution and glycosylation in cells or tissues of several fish species. MHC IIB has been detected in rainbow trout (*Oncorhynchus mykiss*) and channel fish (*Ictalurus punctatus*) peripheral blood leukocytes (PBLs)<sup>[17-18]</sup>, Atlantic salmon (*Salmo salar*) blood leukocytes<sup>[19]</sup>, channel fish clonal T-cell line 28S<sup>[18]</sup>, red snapper (*Lutjanus argentimaculatus*) head kidney, macrophage, and spleen leucocytes<sup>[20]</sup>, along with other tissues (e. g., head kidney, thymus, gill, spleen, hindgut, post kidney, liver, skin, intestine, stomach, heart, and muscle)<sup>[5, 17, 19, 21]</sup>. The native form of MHC IIB is also N-glycosylated, like that of MHC IIA<sup>[5, 17-18, 21]</sup>.

Ayu (sweetfish) (*Plecoglossus altivelis*), the sole member of the Osmeriformes family, Plecoglossidae, is an economically important freshwater fish widely cultured in East Asia, including Japan, China, and Korea. The rapid development of ayu aquaculture is challenged with severe *V. anguillarum* infection that has resulted in problems, both for the production and welfare of this fish in China<sup>[22]</sup>. Thus, the immune molecules of ayu are worthwhile to investigate. In this study, we cloned MHC IIB cDNA from ayu (*PaMHCII B*) and examined its possible role in the immune response. We also investigated the effect of PaMHCII B on monocytes/macrophages (MO/MΦ).

## 1 Materials and methods

### 1.1 Fish rearing and experimental conditions

Ayu (Osmeriformes: Plecoglossidae) samples weighing 40-50 g were obtained from a commercial farm in Fuxi, Ninghai County, Ningbo City, China. The fish were kept in 100 L tanks, equipped with a recirculating system, and filled with filtered freshwater at 20°C ± 0.5°C for two weeks. Only those fish showing no pathological signs were used for the experiments. All the 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, China.

### 1.2 Reagents

Ficoll-Hypaque PREMIUM (1.077 g/mL) was purchased from GE Healthcare (New Jersey, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from Invitrogen (Shanghai, China).

Fluorescein isothiocyanate (FITC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nickel-nitrilotriacetic acid (Ni-NTA) spin column was purchased from Qiagen (Hilden, Germany). Peptide N-Glycosidase F (PNGase F) and endoglycosidase H (Endo H) were purchased from New England BioLabs (Beverly, MA, USA). SYBR premix Ex Taq (Perfect Real Time), *EcoRI*, and *XhoI* were purchased from TaKaRa (Dalian, China). Goat anti-mouse IgG conjugated to HRP was purchased from Biyuntian (Shanghai, China). The enhanced chemiluminescence (ECL) kit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 1.3 Pathogen challenge

The infection with *V. anguillarum* was performed as previously reported<sup>[23]</sup>. Each infected ayu was injected intraperitoneally with 100 μL bacterial suspension ( $1.2 \times 10^5$  colony-forming units (CFU)/mL), whereas the control fish were injected with the same volume of sterile phosphate-buffered saline (PBS). The fish from both groups were sacrificed at 4, 8, 12, and 24 h post infection (hpi). The liver, spleen, trunk kidney, head kidney, gill and intestine were collected, immediately snap-frozen in liquid nitrogen, and preserved at -80°C until total RNA was extracted.

### 1.4 MO/MΦ preparation

The head-kidney-derived MO/MΦ cells were isolated and cultured as previously described<sup>[23]</sup>. Briefly, the ayu head kidney was aseptically extracted. The intermediate leukocyte layer was obtained with Ficoll-Hypaque PREMIUM (1.077 g/mL), following the manufacturer's protocol. After washing with RPMI 1640 medium supplemented with 2% FBS, penicillin (100 μg/mL), streptomycin (100 μg/mL), and heparin (20 U/mL), the cells were cultured overnight in 35 mm dishes at a density of  $2 \times 10^7$ /mL at 24°C in the presence of 5% CO<sub>2</sub>. The non-adherent cells were washed off, and the adherent cells were incubated in a complete medium (RPMI 1640, 4% ayu serum, 6% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) at 24°C in the presence of 5% CO<sub>2</sub>. Over 95% of adherent cells were the MO/MΦ according to morphological characteristics observed after Giemsa staining.

### 1.5 Sequence analysis of *PaMHCII B* cDNA

*PaMHCII B* cDNA sequence was obtained from the transcriptome data of ayu head-kidney-derived MO/MΦ<sup>[24]</sup>, and was verified by sequencing after PCR amplification and cloning in a TA vector. Multiple alignment of *MHCII B* sequences (Table 1) was done using ClustalW (<http://clustalw.ddbj.nig.ac.jp/>), and phylogenetic tree was conducted using MEGA 5.0<sup>[25]</sup>. The PaMHCII B domain architecture was predicted using the SMART program (<http://smart.embl-heidelberg.de/>)<sup>[26]</sup>, and the potential N-glycosylation

Table 1 MHC IIB sequences used in this study

Accession no.	Species	
	Latin name	English name
KX118043	<i>Plecoglossus altivelis</i>	Ayu
ADB12480	<i>Cynoglossus semilaevis</i>	Smooth tongue sole
AAB67872	<i>Ictalurus punctatus</i>	Channel catfish
ACI68945	<i>Salmo salar</i>	Atlantic salmon
BAA94279	<i>Oryzias latipes</i>	Japanese ricefish
NP_001266491	<i>Oreochromis niloticus</i>	Nile tilapia
ACU46021	<i>Epinephelus coioides</i>	Orange-spotted grouper
ACIO5078	<i>Salvelinus alpinus</i>	Arctic charr
NP_001182463	<i>Oncorhynchus mykiss</i>	Rainbow trout
NW_004071367	<i>Takifugu rubripes</i>	Tiger pufferfish
NW_018347190	<i>Boleophthalmus pectinirostris</i>	Great blue-spotted mudskipper
NC_025984	<i>Esox lucius</i>	Northern pike
NW_012224515	<i>Fundulus heteroclitus</i>	Mummichog
NW_017860444	<i>Paralichthys olivaceus</i>	Japanese flounder
NW_018091987	<i>Oncorhynchus kisutch</i>	Coho salmon
NC_036445	<i>Xiphophorus maculatus</i>	Southern platyfish
NC_007119	<i>Danio rerio</i>	Zebrafish
AF084934	<i>Rattus norvegicus</i>	Rat
ABV25623	<i>Gallus gallus</i>	Chicken
BAA02842	<i>Xenopus laevis</i>	African clawed frog
CCE73902	<i>Homo sapiens</i>	Human

sites were predicted using the NetNGlyc1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

1.6 In vitro stimulation of ayu MO/MΦ with V. anguillarum

The stimulation of ayu MO/MΦ with *V. anguillarum* was performed as previously described [27]. After overnight incubation, the adherent cells were incubated in the complete medium for 12 h, and then in an 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 stimulated with PBS. The stimulated and un-stimulated cells were harvested at 4, 8, 12, and 24

hpi, and stored at −80°C before use.

1.7 Real-time quantitative PCR (qPCR)

Total RNA extraction, DNase I digestion, first-strand cDNA synthesis, and qPCR were performed as previously reported [23]. The specific primers for qPCR were designed according to the sequences of *PaMHCII*B, *TNF-α*, *IL-1β*, *IL-10*, *TGF-β*, and 18S rRNA, and are listed in Table 2. The qPCR was performed using SYBR premix Ex Taq in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA). *PaMHCII*B mRNA expression was normalized to that of 18S rRNA (the internal control) using the 2<sup>−ΔΔCT</sup> method.

Table 2 Oligonucleotide primers used in this study

Gene	Primer	Accession No.	Nucleotide sequence (5'→3')	Length (bp)
<i>MHCII</i> B	PaMHCII $\text{B}$ (+)	KX118043	CTGGACAAGCCCATTGAGAA	184
	PaMHCII $\text{B}$ (-)		CAACTCTGTCACCTTAGCCA	
<i>MHCII</i> B	PaMHCII $\text{B}$ ex(+) <sup>a</sup>	KX118043	GGAATTCTATGATTATGAAATGATTAG	579
	PaMHCII $\text{B}$ ex(-) <sup>b</sup>		CCTCGAGTTTACTCCTCTCAGACTCAG	
<i>TNF-α</i>	PaTNFα(+)	JP740414	ACATGGGAGCTGTGTTCTCTC	115
	PaTNFα(-)		GCAAAACACACCGAAAAAGGT	
<i>IL-1β</i>	PaIL-1β(+)	HF543937	TACCGGTTGGTACATCAGCA	104
	PaIL-1β(-)		TGACGGTAAAGTTGGTGCAA	
<i>IL-10</i>	PaIL-10(+)	JP758157	TGCTGCTGCTGCTGTTTATGTGT	73
	PaIL-10(-)		AAGGAGCAGCAGCGGTCAGAA	
<i>TGF-β</i>	PaTGF-β(+)	JP742920	CTGGAATGCCGAGAACAAAT	101
	PaTGF-β(-)		GATCCAGAACCTGAGGGACA	
18S rRNA	18S rRNA(+)	FN646593	GACACGGAAAGGATTGACAG	119
	18S rRNA(-)		CGGAGTCTCGTTCGTTAT	

<sup>a</sup>and <sup>b</sup>: The underlined sequence represents *Eco* RI (in PaMHCII $\text{B}$ ex(+)) and *Xho* I (in PaMHCII $\text{B}$ ex(-)) restriction sites, respectively



1.8 Expression of PaMHCII B in prokaryotic host and antibody preparation

Table 2 shows the primer pair PaMHCII Bex ( + ) and PaMHCII Bex ( - ) designed to amplify the extracellular region ( residues 23-215 ) of PaMHCII B ( PaMHCII Bex ) without the signal peptide. The amplicons were digested with *Eco* R I and *Xho* I , directionally cloned into the pET28a vector, and transformed into *Escherichia coli* BL21 ( DE3 ). After IPTG induction, the recombinant PaMHCII Bex protein

with 6 × His-tag ( rPaMHCII Bex ) was purified using a Ni-NTA spin column, following the manufacturer ’ s protocol. The purity of rPaMHCII Bex was confirmed using SDS-polyacrylamide gel electrophoresis ( SDS-PAGE ). Finally, the purified rPaMHCII Bex was inoculated into mice to produce the antiserum.

1.9 Deglycosylation

The denatured protein from ayu MO/MΦ was digested with PNGase F and Endo H as previously described [ 5 ] to determine whether native PaMHCII B is

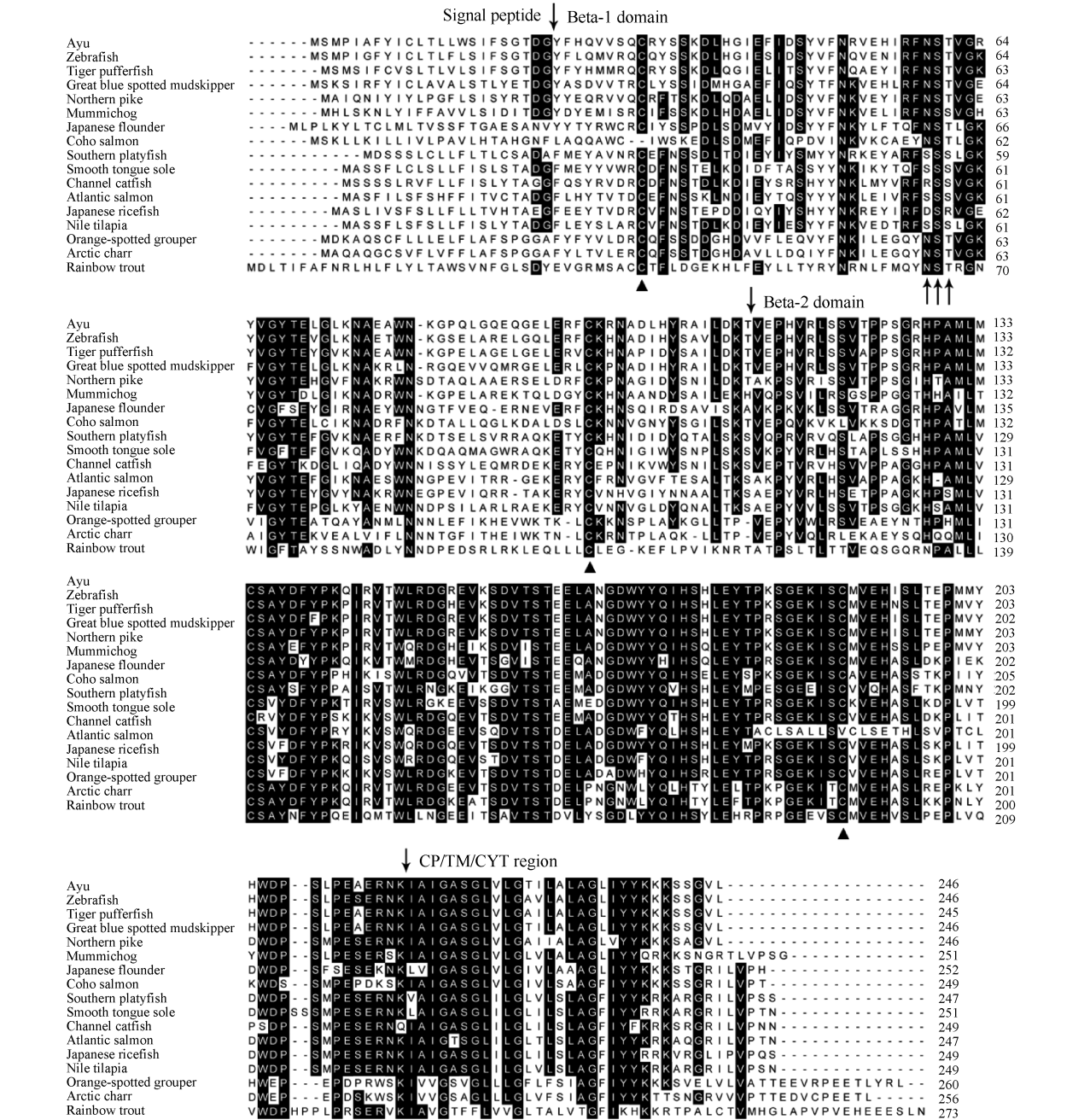


Fig. 1 Multiple alignment of the amino acid sequences of PaMHCII B and other fish MHC II B The threshold for shading is >60% ; the gray shading, black shading, and “-” represents similar residues, identical residues, and gap in the alignment, respectively. “▲” represents the conserved cysteine residues forming the disulfide bridges and “↑” represents an N-glycosylation site. The accession numbers of the sequences used are listed in Table 1

N-glycosylated. In brief, 500  $\mu\text{g}$  of proteins obtained from ayu MO/M $\Phi$  lysate were boiled for 10 min in denaturing buffers (0.5% SDS, 1%  $\beta$ -mercaptoethanol). Thereafter, 2 500 units of Endo H was added to the sample in sodium citrate buffers (pH 7.5) and the mixture was incubated overnight at 37 $^{\circ}\text{C}$ . A second overnight digestion was done at 37 $^{\circ}\text{C}$  with 20 U of PNGase F in sodium phosphate buffers (pH 7.5) containing 1% NP-40.

1.10 Western blot

Protein samples from MO/M $\Phi$  samples were analyzed using SDS-PAGE and Western blotting as described by Li *et al.* [28]. The antibody against PaMHCIIB was used as the primary antibody at a dilution of 1:500, and goat anti-mouse IgG conjugated to HRP was used as the second antibody at a dilution of 1:5 000. The proteins were visualized using an ECL kit. Changes in relative band intensity were analyzed using the NIH ImageJ program and normalized against  $\beta$ -actin.

1.11 Phagocytosis assay

*Escherichia coli* DH5 $\alpha$  cells in the mid-logarithmic phase of growth, were labeled with FITC. Ayu MO/M $\Phi$  were blocked with anti-PaMHCIIBex IgG (200

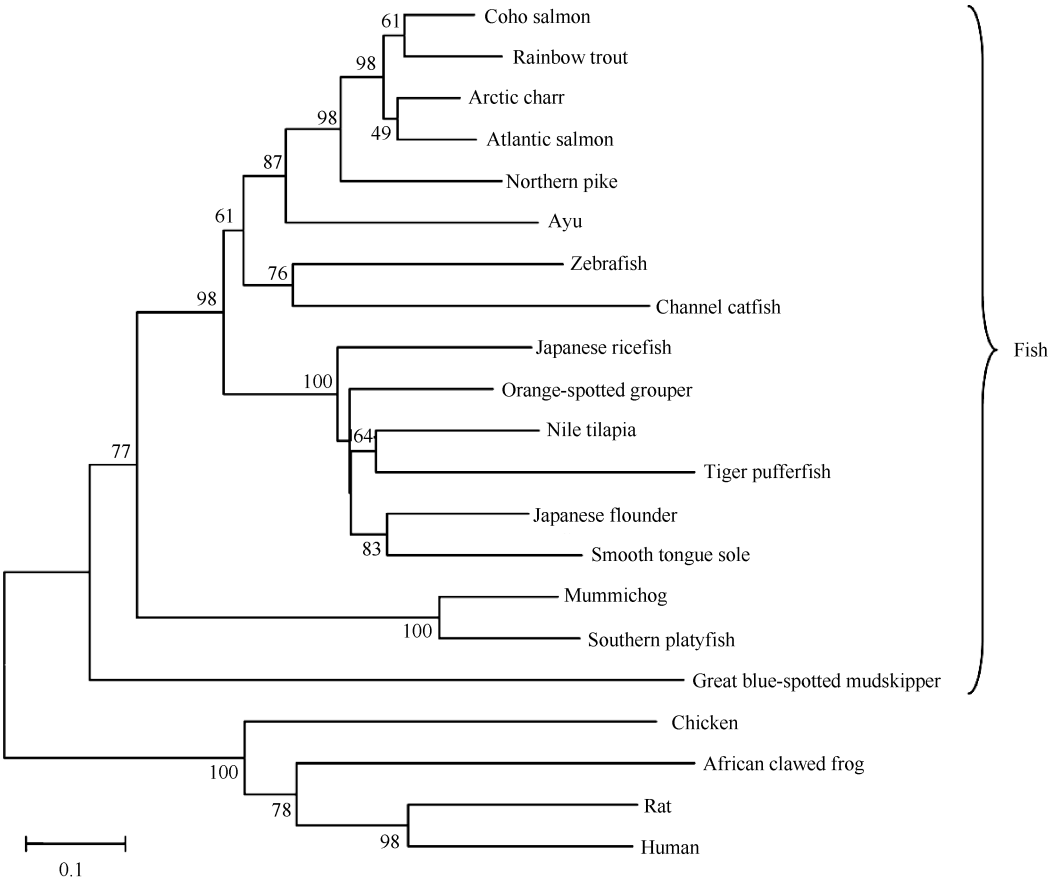
$\mu\text{g}/\text{mL}$ ) for 30 min for PaMHCIIB neutralization. A mouse isotype IgG (200  $\mu\text{g}/\text{mL}$ )-treated group was used as control. Heat-killed FITC-DH5 $\alpha$  cells were added at an MOI of 20. After extensive washing with sterile PBS to remove the extracellular particles, the cells were further incubated for 30 min. Trypan blue (0.4%) was used to quench the extracellular fluorescence. Thereafter, the cells were collected and resuspended in FACS buffers (PBS, 0.2% BSA, 0.1% sodium azide). The MO/M $\Phi$  bacterial uptake was examined by using a Gallios flow cytometer (Beckman Coulter, Miami, USA).

1.12 MO/M $\Phi$  cytokine mRNA expression analysis

PaMHCIIBex neutralization was performed as described above. The cells were then infected with live *V. anguillarum* at an MOI of 2, collected at 4, 8, 12, and 24 hpi, and used to measure the changes in mRNA expression of *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-10*, and *TGF- $\beta$* . The PBS-treated group was used as control.

1.13 Statistical analysis

Results are presented as mean  $\pm$  SD. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 13.0 (SPSS Inc., Chicago, USA). Statistical significance was determined at  $P < 0.05$ .



**Fig. 2 Phylogenetic (neighbor-joining) analysis of the complete MHC IIB amino acid sequences using MEGA5.0 program** Values at the nodes indicate the percentage of trees (>60%) that included the grouping (after bootstrapping for 1 000 replicates). The scale bars show the number of substitutions per base. Accession numbers of sequences used are listed in Table 1

2 Results

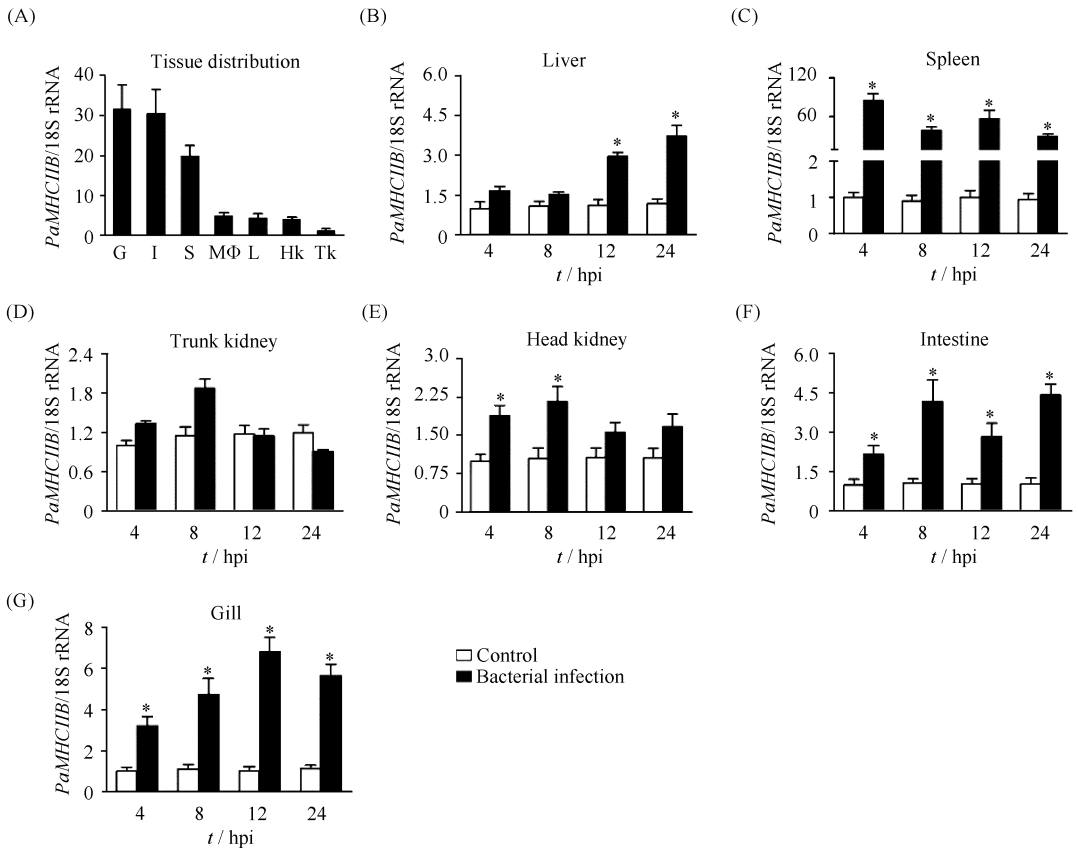
2.1 Analysis of the PaMHCII B cDNA sequence

The 920 bp *PaMHCII B* cDNA contained an open reading frame of 756 bp that coded a 251-amino acid (aa) containing protein with a putative molecular weight (MW) of 28. 23 kD and a theoretical isoelectric point (pI) of 6. 55. The ATG initiation codon was from 32-34 nucleotides (nts), and the 3'-UTR was 133 bp long. The nucleotide sequence of *PaMHCII B* cDNA was deposited in the GenBank (accession number KX118043).

Results of putative aa sequence analysis revealed that *PaMHCII B* had a 22-aa signal peptide (SP), two extracellular domains ( $\beta$ 1: residues 23-110;  $\beta$ 2: residues 111-215), and a conserved connecting peptide/transmembrane/cytoplasmic (CP/TM/CYT) domain (residues 216-251) (Fig. 1), all of which are the typical characteristics of the MHC class II. One potential N-linked glycosylation site was predicted in the  $\beta$ 1 domain at position Asn 58 (Fig. 1). The

*PaMHCII B* sequence also contained four cysteine residues that formed the intra-domain disulfide bridges ( $\beta$ 1 domain: residues 31 and 95;  $\beta$ 2 domain: residues 133 and 189) (Fig. 1), as in other fish species. Moreover, the conserved GxxGxxxGxxxxxxG motif (where x is any hydrophobic residue other than Gly) was observed in the TM region (Fig. 1).

The amino acid sequence alignments indicated that the *PaMHCII B* sequence had greater similarity to those of fish MHC IIB (32. 27% to 65. 04%), and shared the highest aa identity (65. 04%) with Arctic charr (*Salvelinus alpinus*) MHC IIB. The phylogenetic analysis showed that the MHC IIB sequences of ayu and other fish were grouped together, forming a fish MHC IIB cluster distinct from the mammalian-amphibian-bird MHC IIB cluster (Fig. 2). *PaMHCII B* was observed to be most closely related to Arctic charr MHC IIB, but was more distant from MHC IIBs of Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), and northern pike (*Esox lucius*) (Fig. 2),



**Fig. 3** Quantitative real-time polymerase chain reaction analysis of *PaMHCII B* transcripts in healthy fish or in fish challenged with *V. anguillarum* (A) *PaMHCII B* transcripts in different tissues from healthy ayu. G: gill, I: intestine, S: spleen, M: MO/M $\Phi$ , L: liver, Hk: head kidney, Tk: trunk kidney. (B-G) *PaMHCII B* transcripts in different tissues of ayu challenged with *V. anguillarum*. Each infected ayu was injected intraperitoneally with 100  $\mu$ L bacterial suspension [ $1.2 \times 10^5$  CFU/mL], whereas the control group was injected with the same volume of sterile PBS. The samples were collected at different time points after *V. anguillarum* challenge (x-axis). The *PaMHCII B* transcript levels were normalized to the expression levels of ayu 18S rRNA gene. Data are expressed as means  $\pm$  SD ( $n=4$ ). \*  $P < 0.05$ , compared with the control group at each time point

reflecting the currently accepted phylogenetic relationship.

2.2 *PaMHCIIB* mRNA expression is induced by *V. anguillarum* infection

*PaMHCIIB* mRNA expression levels in the ayu tissues and cells after *V. anguillarum* challenge were determined using qPCR. *PaMHCIIB* transcripts were detected in all tested tissues, and MO/MΦ of healthy ayu; the expression levels were higher in the gill, intestine, and spleen (Fig. 3A). Upon *V. anguillarum* challenge, the *PaMHCIIB* transcripts in all of the tested tissues except for the trunk kidney were significantly increased [in liver at 12 and 24 hpi (Fig. 3B), in spleen at all the time points (Fig. 3C), in head kidney at 4 and 8 hpi (Fig. 3E), in intestine at all the time points (Fig. 3F), and in gill at all time points (Fig. 3G)]. Compared with the control, the *PaMHCIIB* mRNA expression level in the liver was gradually upregulated during the 4-24 h period, increasing by 1.97-fold until 12 hpi and reaching a peak of 3.18-fold at 24 hpi (Fig. 3B). The *PaMHCIIB* mRNA expression level in the spleen, head kidney, intestine, and, gill was rapidly upregulated at 4 hpi, and reached maximum at different time points (for spleen, 85.18-fold at 4 hpi; for head kidney, 2.06-fold at 8 hpi; for intestine, 4.21-fold at 24 hpi; and for gill, 6.81 at 12 hpi) (Fig. 3C, E, F, and G). Notably, *V. anguillarum* infection resulted in a much higher induction of *PaMHCIIB* mRNA levels in the spleen than those in other tissues (Fig. 3C).

2.3 Prokaryotic expression and confirmation of N- glycosylation of *PaMHCIIB*Bex

r*PaMHCIIB*Bex was overexpressed in BL21 (DE3) cells transformed with the pET28a-*PaMHCIIB*Bex plasmid (Fig. 4 A, lane 2). The observed molecular mass of the recombinant protein determined using SDS-PAGE was about 26 kD, which is consistent with the expected size (22.09 kD *PaMHCIIB*Bex + 3.84 kD His-tag) (Fig. 4 A, lane 2). The insoluble r*PaMHCIIB*Bex was extracted from inclusion bodies using urea, and was re-natured. The target protein was further purified on a Ni-NTA column. The purity of r*PaMHCIIB*Bex was also analyzed using SDS-PAGE, which showed a single band at the position expected from its molecular size (Fig. 4 A, lane 3). The Western blotting analysis showed a single protein band at about 26 kD for the recombinant protein (Fig. 4B, lane 1). The observed molecular mass of the native *PaMHCIIB* in MO/MΦ was approximately 35 kD (Fig. 4B, lane 2), which was reduced to 32 kD after Endo H and PNGase F digestion (Fig. 4B, lane 3).

2.4 *PaMHCIIB* expression at mRNA and protein levels in MO/MΦ upon *V. anguillarum* infection

We investigated *PaMHCIIB* mRNA and protein expression levels in healthy and *V. anguillarum*-infected

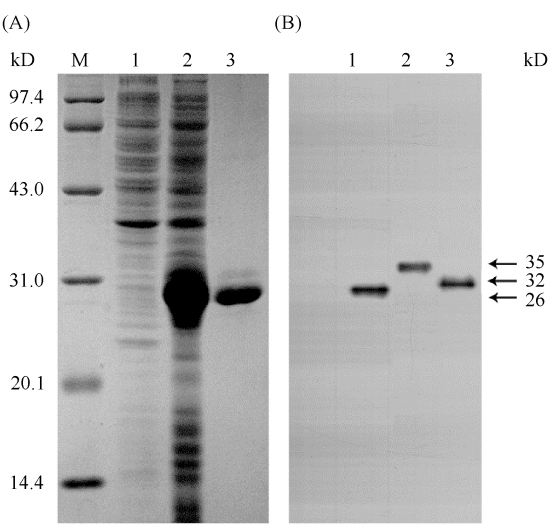


Fig. 4 Prokaryotic expression of *PaMHCIIB* in *E. coli* and confirmation of *PaMHCIIB* N-glycosylation in the native ayu MO/MΦ (A) SDS-PAGE analysis of the extracellular *PaMHCIIB* peptide expressed in the prokaryotic host. Lane M: protein molecular weight standards; 1: before IPTG induction; 2: after IPTG induction; 3: purified recombinant protein. (B) Western blotting analysis of recombinant, native, and PNGase F- and Endo H-digested *PaMHCIIB* 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, and digested with PNGase F and Endo H

ayu MO/MΦ. qPCR showed that *PaMHCIIB* mRNA expression was significantly upregulated in MO/MΦ upon *V. anguillarum* infection at all the time points and reached the peak at 12 hpi (3.35-fold) (Fig. 5 A). Western blotting analysis showed that the *PaMHCIIB* protein level also significantly increased in MO/MΦ upon *V. anguillarum* infection at 12 and 24 hpi and reached the peak at 24 hpi (3.19-fold) (Fig. 5B).

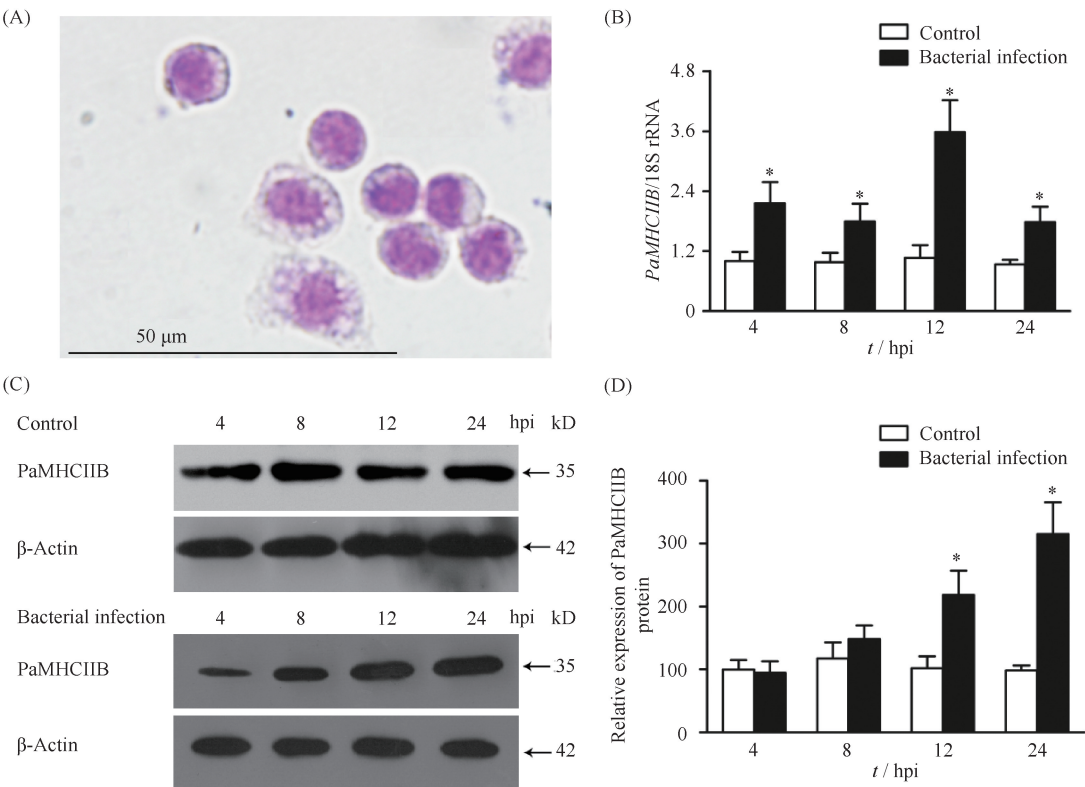
2.5 *PaMHCIIB* mediates the phagocytosis of ayu MO/MΦ

Results of the phagocytosis assay showed that the ability of MO/MΦ to phagocytize *E. coli*-FITC changed significantly after *PaMHCIIB* neutralization by anti-*PaMHCIIB*Bex IgG (Figs 6 A and 6B). After *PaMHCIIB* neutralization, the MO/MΦ phagocytosis of *E. coli*-FITC decreased by 72.26% (Fig. 6B).

2.6 *PaMHCIIB* affects the cytokine mRNA expression in ayu MO/MΦ

The mRNA expression levels of *TNF-α*, *IL-1β*, *IL-10*, and *TGF-β* were evaluated to determine how *PaMHCIIB* affected the cytokine mRNA expression in ayu MO/MΦ. Ayu MO/MΦ was infected with *V. anguillarum* following treatment with anti-*PaMHCIIB*Bex or isotype IgG. qPCR analysis revealed that the expression of the four cytokines were all significantly downregulated in the anti-*PaMHCIIB*Bex IgG-treated





**Fig. 5** *PaMHCII B* expression in *V. anguillarum*-infected MO/MΦ isolated from ayu (A) MO/MΦ from healthy ayu on the slides were fixed with methanol, stained with Giemsa, and examined using light microscopy. (B) qPCR was used to analyze *PaMHCII B* mRNA expression in MO/MΦ. The cells in an antibiotic-free medium were infected with live *V. anguillarum* at an MOI of 2. The control group was stimulated with PBS. The cells were collected at 4, 8, 12, and 24 hpi. *PaMHCII B* transcript levels were normalized to that of 18S rRNA.  $n = 5$ . (C) Western blotting was conducted to analyze *PaMHCII B* protein expression using an antibody specific for *PaMHCII B*. Histograms displaying the changes in relative band intensity of *PaMHCII B* in cells collected at 4, 8, 12, and 24 hpi. *PaMHCII B* protein expression was normalized to that of  $\beta$ -actin. Data are expressed as the mean  $\pm$  SD.  $n = 3$ . \*  $P < 0.05$ , compared with the control group at each time point.

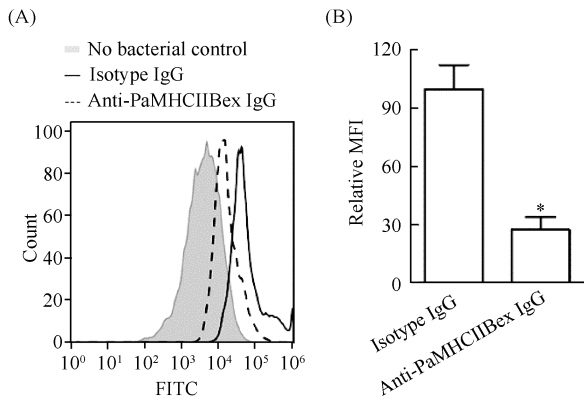
MO/MΦ compared with that in the isotype IgG-treated MO/MΦ during the 8-24 h period (Fig. 6 A-D). Significant down-regulation was observed for *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-10*, and *TGF- $\beta$*  at 8 hpi (0.23-, 0.41-, 0.51-, and 0.20-fold, respectively, compared with the isotype IgG-treated group) (Fig. 6 A-D).

3 Discussion

In the present work, we cloned the ayu *MHC class II B* gene (*PaMHCII B*) and analyzed its structure. The deduced peptide sequence contained the common structural features of MHC IIB, including the SP, CP/TM/CYT, and extracellular ( $\beta 1/\beta 2$ ) domains, consistent with those present in other species [7-9, 11, 13, 15-16, 20-21]. Moreover, the TM region of *PaMHCII B* contained the GxxGxxxGxxxxxxG motif, shared by mammals, birds, amphibians, and fishes [9, 16, 20, 29-31]. *PaMHCII B* has only one potential N-glycosylation site, as in other teleost [7, 9, 11, 13, 16, 20] except the miiuy croaker [15]. Similarly, human, bird, and amphibian MHC IIB proteins have only one N-glycosylation site [29, 31-32]. Overall, the high degree of

structural conservation in MHC IIB suggests that this protein class possibly plays a crucial function in vertebrates.

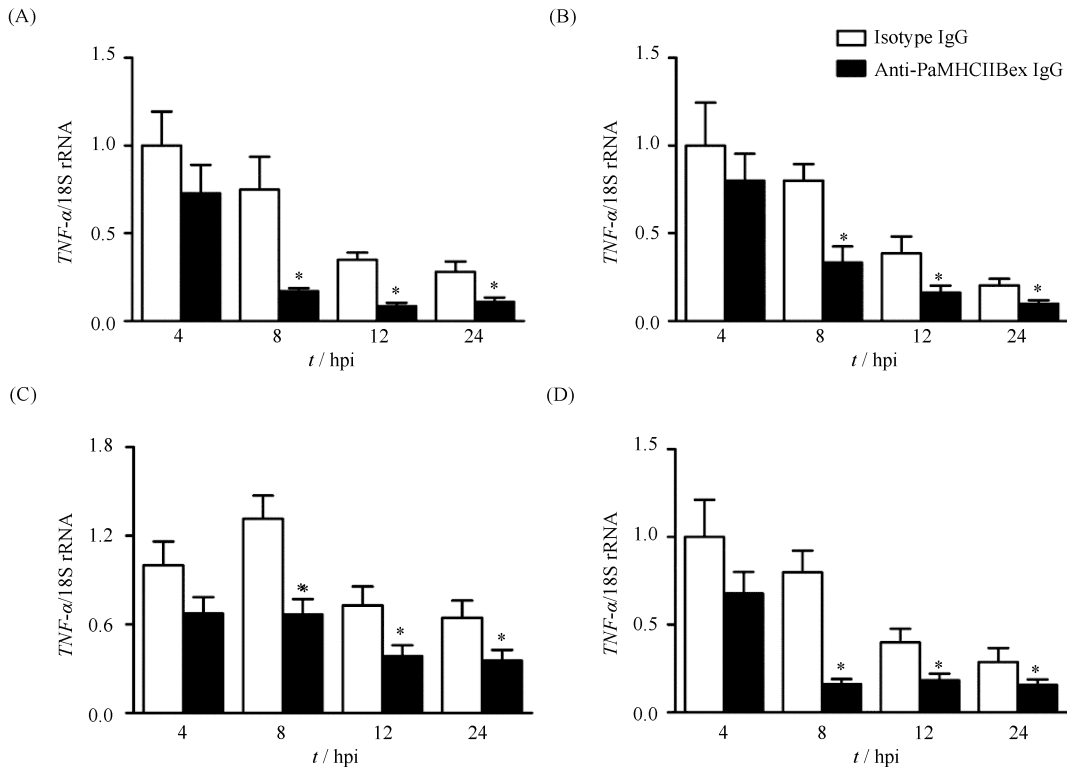
We observed that *PaMHCII B* mRNA was widely expressed in all the tested immune tissues and MO/MΦ, with especially high transcription in the gill, intestine, and spleen, consistent with the findings in other teleost. In Nile tilapia, *MHC IIB* mRNA expression level was highest in the gill, followed by the kidney, intestine, and spleen [16]. Similarly, *MHC IIB* mRNA expression in the red spotted grouper (*Epinephelus akaara*) was high in the head kidney, spleen, gill, and intestine [33]. Moreover, in rainbow trout, the *MHC IIB* mRNA was expressed in head-kidney macrophage and non-clonal macrophage-like cell line (RTS11) [34-35]. Until now, *MHC IIB* transcripts tend to increase in bacterial infection or immune stimulation in several fish including large yellow croaker [7], blunt snout bream [9], spotted halibut [11], Nile tilapia [16], and golden pompano (*Trachinotus ovatus*) [36]. In this study, *MHC IIB* transcripts and proteins were significantly induced by



**Fig. 6 Effect of PaMHCIIB neutralization on ayu MO/MΦ phagocytosis** Anti-PaMHCIIBex IgG was used to block PaMHCIIB on MO/MΦ, whereas the isotype IgG was used as control. (A) The bacterial uptake was determined using a Gallios flow cytometer. (B) The relative mean fluorescence intensity (MFI) of anti-PaMHCIIBex IgG-treated group was presented as fold change relative to the isotype IgG-treated control, which was assigned a value of 100. Data are expressed as means  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$ , compared with the isotype IgG-treated control.

*V. anguillarum* infection, which is in accordance with the results mentioned above. *MHCIIA* transcripts were also dramatically induced by bacterial infection or immune stimulation in some fish, such as large yellow croaker [7], blunt snout bream [9], and ayu [37]. Our data, combined with those obtained in previous studies, revealed a rapid early response of MHC IIB to immune stimulation or pathogen infection, with obvious transcriptional or protein upregulation during early inflammation [7, 9, 11, 16, 36], revealing that MHC IIB plays a crucial role in protecting fish against pathogen infection like *MHCIIA*.

In the present work, Western blotting analysis revealed that PaMHCIIB was definitely N-glycosylated and constituted about 10% of the glycoprotein's total mass. In agreement with our findings, the native MHC IIB from rainbow trout [5, 17], catfish [18], Atlantic salmon [19], and stickleback [21] were also reported to have N-linked modification. For example, the MW of native MHC IIB from rainbow trout PBLs was approximately 34 kD, which is higher than the predicted MW of the mature peptide, and was about 31 kD after deglycosylation [17].



**Fig. 7 Effects of PaMHCIIB neutralization on the mRNA expression of four cytokines in *V. anguillarum*-challenged ayu MO/MΦ** Real-time quantitative PCR was used to measure the mRNA expression of *TNF-α* (A), *IL-1β* (B), *IL-10* (C), and *TGF-β* (D). The MO/MΦ cells were pretreated with anti-PaMHCIIBex IgG or the isotype IgG for 30 minutes, and then *V. anguillarum* was added to the cell culture media at an MOI of 2. The cells were collected at different time points. The transcript levels of these four cytokines were normalized to levels of 18S rRNA. Data are expressed as the means  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$ , compared with the isotype IgG-treated control at different time points.

Prior to this study, knowledge about the immune response of MHC II in MO/MΦ of fish was scant. Herein, we found that PaMHCII B neutralization significantly restrained ayu MO/MΦ phagocytosis and suppressed the expression of cytokines (*IL-1β*, *TNF-α*, *IL-10*, and *TGF-β*) in ayu MO/MΦ after infected by *V. anguillarum*. Our previous data have also shown that PaMHCII A performed almost the same function<sup>[37]</sup>. These results demonstrate that ayu MHC II likely contributes to host resistance to pathogen infection in ayu MO/MΦ. In mammals, MHC II also participates in phagocyte activation, cytokine production, cell adhesion, apoptosis, and proliferation and differentiation of B lymphocytes<sup>[38-40]</sup>. Earlier studies have shown that stimulation with staphylococcal exotoxin B and toxic shock syndrome toxin-1 upregulated the transcription of *IL-1β* and *TNF-α* in the human peripheral blood monocytes (PBMC) and THP-1 monocytic cells<sup>[41]</sup>. Moreover, MHC II deficiency diminished the secretion of lipopolysaccharide (LPS)-induced inflammatory cytokines (*TNF-α*, *IL-1*, or *IL-8*) in PMBCs and THP-1 monocytic cells<sup>[38]</sup>, in accordance with our observations. Therefore, the MHC II-mediated immune activation by ayu MO/MΦ greatly contributes to our better understanding of the MHC II function in mammals, but future research is required to investigate the potential mechanisms underlying the relationship between ayu MHC II and MO/MΦ function.

We have characterized a novel MHC class II B gene from ayu. The native form of PaMHCII B has N-glycosylation. *PaMHCII B* transcripts were remarkably enhanced in various immune tissues during bacterial infection, and *PaMHCII B* transcripts and protein were both significantly upregulated in ayu MO/MΦ infected with *V. anguillarum*. Our results suggest that PaMHCII B neutralization can inhibit phagocytosis and the expression of cytokine mRNAs in ayu MO/MΦ. We hope that this work would be useful in further elucidating the role of fish MHC class II.

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