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Molecular characterization and expression of CD96 in Nile tilapia (*Oreochromis niloticus*) in response to different pathogens stimulus

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ABSTRACT

CD96 is one of the immune superfamily members and it is identified as a T cell-specific receptor. In this study, we cloned a CD96 from Nile tilapia (*Oreochromis niloticus*, named OnCD96). The open reading frame of *OnCD96*was 1371 bp, encoding a protein of 456 amino acids. Sequence alignment analysis indicated that OnCD96 contained two Ig-like domains in entodomain, proline-rich motif, and ITIM motif in the cytoplasm. Subcellular localization studies showed that OnCD96 was distributed mainly in the cytoplasm and on the cytoplasmic membrane. In healthy tilapia, *OnCD96* was distributed in all the tested tissues and relatively higher in the liver. After *Streptococcus agalactiae* infection, the expression of *OnCD96* in the thymus, brain, and spleen reached its peak at 72 h, 48 h, and 24 h, respectively. After stimulation with poly I:C, the expression of *OnCD96* in thymus and spleen reached its peak at 6 h and 24 h respectively. While, the expression in the brain had double peaks, which were at 24 h and 96 h. After stimulation with Keyhole limpet hemocyanin (KLH), a classical T cell-dependent antigen, the expression of *OnCD96* was significantly up-regulated in blood and head kidney. Moreover, compared with the first challenge, *OnCD96* was up-regulated earlier after the second challenge. Yeast two-hybrid assay indicated that there might be interaction between OnCD96 and OnNecl5. These results suggested that OnCD96 plays an important role during pathogens infection, and the interaction between CD96 and Necl5 is conserved in Nile tilapia.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is an important economical fish and widely farmed in all parts of the world. However, since tilapia germplasm degradation and environmental pollution, the outbreak of various diseases caused huge economic losses to the tilapia farming industry (Amal and Zamri-Saad, 2011; Garcia et al., 2010). Due to the side effects of drug control, studies on the prevention and treatment of fish disease based on immune mechanism has become a hot spot. In the early stages of our laboratory, we have focused on the research of T cell immune mechanisms of tilapia and many molecules such as CD48 (Wang et al., 2020), CD2 (Bei et al., 2016), Lck (Gan et al., 2016a), ZAP-70 (Gan et al., 2016b) and CD59 (Gan et al., 2015) are involved in T cell activation.

In mammals, CD96 was identified as a T cell-specific receptor, also known as Tactile (T cell activation increased late expression), which can highly upregulate T cell activation. In 1992, human CD96 (hCD96) was first cloned and expressed by Wang et al. (1992). The protein coded by CD96 gene belonged to the immunoglobulin superfamily. CD96 is mainly expressed in T cell, maintaining low levels on resting T cell and NK cell but strongly up-regulated after T cell and NK cell activation. However, peripheral B cells do not express CD96. They are usually not expressed in non-hematopoietic cell types such as cancer cells or fibroblasts, except for certain intestinal epithelial cells (Gramatzki et al., 1999). Structurally, there are three Ig domains in the entodomain of CD96, the first one is IgV and the latter two are IgC. There are 24 amino

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acid residues in the transmembrane region, 15 potential N-glycosylation sites outside the membrane, and 6 cysteine residues that can form 3 disulfide bonds. The intracellular region of CD96 has no motif that binds the PDZ domain but has a proline-rich region and an ITIM-like motif (Amal and Zamri-Saad, 2011). CD96 shares the same ligand Necl5 with CD226 and TIGIT (Fuchs et al., 2004), they form an interactive network: only CD226 was reported to activate T and NK cells (Susan et al., 2008; Shibuya et al., 1996; Akiko et al., 2008). TIGIT (Joller et al., 2011) and CD96 interact with Necl5 to transmit inhibitory signals. But the interactive network is quite complex, many factors are involved in the net inhibition/activation (Stanietsky and Mandelboim, 2010; Martinet and Smyth, 2015; Georgiev et al., 2013; Chan et al., 2012). The regulatory network established by Necl5-family has received more and more attention because of its important role in immune surveillance. The interaction between CD96 and Necl5 plays an indispensable role in this network. CD96 has been regarded as an important molecular marker of acute myeloid leukemia stem cells (Hosen et al., 2007; Burger et al., 1999), also has been reported to be associated with C syndrome (Opitz Trigonocephaly) (Kaname et al., 2007).

Given that CD96 plays an important role in mammalian T cell immunity, it is unclear in fish. This study conducted a preliminary study on Nile tilapia, firstly, the cDNA of *CD96* was cloned from Nile tilapia and was over-expressed in HEK293 T cells to detect its intracellular localization. Then the mRNA expressions of OnCD96 in healthy tilapia and under different stimuli were determined *in vivo*. Finally, we detected the interactions between OnCD96 and OnNecl5 by yeast two-hybrid. These findings may help us to understand the role of OnCD96 in the immune response of Nile tilapia.

2. Materials and method

2.1. Fish, immunization, and sampling

Fish (average weight of 100 ± 10 g) were bought from a commercial farm in Zhanjiang, Guangdong province, China. Before the experiment, fish were reared in Fiber-reinforced plastic tanks (1000 L each) under 28 ± 2 ℃ for 4 weeks.

In the study of *OnCD96* tissue expression, three healthy fish were randomly selected for tissue extraction, namely gill, kidney, muscle, skin, blood, liver, intestine, brain, thymus, and spleen ten tissues were collected. Immediately after sampling, all tissue samples were frozen with liquid nitrogen and transferred to -80 ℃ until used.

To study the response of *OnCD96* to bacterial and viral stimuli, 90 fish were randomly divided into 3 groups and injected with PBS, *S. agalactiae*, and poly I:C. *S. agalactiae* (*Streptococcus agalactiae*, ZQ0910) was used for immunostimulation and cultured following the procedures described in the literature (Wang et al., 2012). For the test groups, formalin-inactivated *S. agalactiae* suspension (1×10^7 cells·mL^{-1}), poly I:C (Sigma, USA) (0.2 mg·mL^{-1}) were injected intraperitoneally respectively, while the control group was injected with sterilized PBS (100 μL) intraperitoneally. At time points of 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h post-immunization, tissues were collected from the control and the test groups. All the above samples were in triplicates. Immediately after sampling, all tissue samples were frozen with liquid nitrogen and transferred to -80 ℃ for future research.

KLH is a classical T cell-dependent (TD) antigen, this study determined the dynamic trends of *OnCD96* after KLH stimuli to explore whether OnCD96 participated in host defense against T cell-dependent pathogen infection. 100 fish were randomly divided into 2 groups and injected with PBS and KLH respectively. KLH (Sigma, USA) was diluted to 1 μg·μL^{-1} with sterilized PBS, the test group was injected with 100 μL of KLH, and the control group was injected with 100 μL of sterilized PBS. The first immunization was done on the 0 day and the second immunization was done on the 28th day. The tissues were collected at 1, 3, 5, 7, 14, 21, and 28 days post-first and post-second challenge from each group. All the above samples were in triplicates. Immediately after sampling, all tissue samples were frozen with liquid nitrogen and transferred to -80 ℃ for future research.

All experiments were conducted according to the principles and procedures of the Laboratory Animal Management Ordinance of China.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from all the above samples with TransZol Up (*TransZol* Up Plus RNA Kit, Trans, China), and the first-strand cDNA was synthesized according to the reverse transcription kit instructions (EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix, Trans, China). Then the first-strand cDNA was stored at -20 ℃ until used.

2.3. Cloning and sequence analysis of OnCD96

The specific primers were designed with the software Primer Premier 5.0, based on the sequence of *OnCD96* mRNA (GenBank Accession XM_005472248.4) in NCBI. All the primers used in this study were synthesized by Guangzhou Sangon and listed in Table 1. The PCR reaction conditions were as follows: 94℃ for 5 min; 94℃ 30 s; 55℃ 30 s, 72℃ 1 min 25 s, 33 cycles; 72℃ 10 min; 4℃ forever. All the PCR products were ligated to pMD18-T vector then transformed into competent *Escherichia coli* cells. Finally, positive clones were sent to Guangzhou Sangon for sequencing, the results were assembled using Seqman.

The sequence analysis, multiple-sequence alignment, and phylogenic tree analysis were consistent with the methods described in Wang's article (Wang et al., 2020).

2.4. Subcellular localization analysis of OnCD96

OnCD96 was ligated to the pDsRed-Monomer-N1 vector using NheI and ApaI restriction sites, the primers were listed in Table 1. HEK-293 T cells were cultured in high glucose Dulbecco's modified Eagle's medium, which contained 10 % fetal bovine serum (Gibco, USA) and 5 % CO_2 incubator at 37 ℃. HEK-293 T cells were seeded into a 6-well plate that has been placed in sterile coverslips in advance. After 18 h of cell culture, empty pDsRed-Monomer-N1 and pDsRed-OnCD96 were transfected into HEK-293 T cells according to the instructions of Lipofectamine™ 3000 reagent (Invitrogen, USA). After 24 h of transfection, washing of HEK-293 T cells with PBS, fixation, and nuclear staining was performed using 4 % paraformaldehyde and DAPI (1 μg·ml^{-1}) respectively. Finally, the cells were rinsed again with PBS, fixed with 50 % glycerol, and observed with a fluorescence microscope (Leica, Germany).

2.5. Spatiotemporal expression feature of OnCD96 mRNA

The spatiotemporal expression of *OnCD96* in pre- and post-immunized tissues were measured by qRT-PCR technology with a LightCycler® Real-time system (ROCHE). The β-actin gene was used as

Table 1
Primers used in this study.

Primers	Sequence (5′-3′)	Purpose
On-CD96-1F	ATGAGTTTGGGGACCT	ORF cloning
On-CD96-1371R	TCATATTTGAGTCTCTGTTAA	
On-CD96-1S	CTAGCTAGCATGAGTTTGGGGACC	protein expression
On-CD96-1368A	TATGGGCCCCTATTTGAGTCTCTGT	
On-CD96-1F	CCGGAATTCGCTGAGTTGTTCCAT	Yeast two-hybrid
On-CD96-1056R	CCGCTCGAGCCGGTGCCGTATCCTT	
On-Necl5-1F	ATGCCATGGAGAAATTCTCTATGATC	
On-Necl5-971R	CGCGGATCCTTAAGAGCATGCTCCTGA	
On-CD96-1043F	CAGCAACGCCAGAAGAATACA	qPCR
On-CD96-1289R	TACTTCACTGGAGGTGGAGGG	
β-actin-S	AACAACCACACACCACACATTTC	qPCR
β-actin-A	TGTCTCCTTCATCGTTCCAGTTT	

an internal control to normalize the potential variations in RNA loading.

Each reaction system was 10 μL volume, consisting of 0.5 μL of each primer (10 mM), 0.5 μL of template, 5 μL of SYBR® Select Master Mix, and 3.5 μL of PCR-grade water. Usually, the water was premixed with primers, while the template premixed with Master Mix. PCR amplification program was as follows: 94 °C for 5 min; 94 °C for 10 s, 60 °C for 1 min, 40 cycles. At the end of each PCR reaction, melting curve analysis was performed at 95 °C to confirm the production of single products. Each sample was in triplicate. The relative expression of OnCD96 was calculated using the $2^{-\Delta\Delta Ct}$ method. One-way analysis of variance was performed using SPSS 20.0 software.

2.6. Yeast two-hybrid assay

The potential interactions between OnCD96 and OnNecl5 proteins were detected by yeast two-hybrid assay. OnCD96 was ligated to pGADT7 vector to act as a prey protein, while OnNecl5 was ligated to pGBKT7 vector to act as a bait protein, the primers were listed in the Table 1. First, competence Y2HGold cells were prepared according to the Matchmaker Gold Yeast Two-Hybrid System User Manual (Takara, Japan). Then, the recombinant plasmids for autoactivation, pGADT7-OnCD96 and pGBKT7 plasmids were tested, as well as pGBKT7-OnNecl5 and pGADT7 plasmids were co-transfected to competence Y2HGold cells respectively. Yeasts were grown on the corresponding plates (SD/-Leu, SD/-Leu/X, SD/-Leu/X/A; SD/-Trp, SD/-Trp/X, SD/-Trp/X/A) for 3–5 days at 30 °C. After confirming that the recombinant plasmids had no self-activation, the experimental group was co-transfected with pGADT7-OnCD96 and pGBKT7-OnNecl5, while the positive and negative control groups were co-transfected with pGBKT7-53 and pGADT7-T plasmids, as well as pGBKT7-Lam and pGADT7-T plasmids respectively. Each group of yeasts was spread on three kinds of plates: double dropout medium supplemented with X-a-Gal (SD/-Leu/-Trp/X), triple dropout medium supplemented with X-a-Gal and Aureobasidin A(SD/-His/-Leu/-Trp/X/A), quadruple dropout medium supplemented with X-a-Gal and Aureobasidin A(SD/-His/-Ade/-Leu/-Trp/X/A). The turning of a single colony into blue, indicated that there was an interaction between the proteins.

3. Results

3.1. Cloning and the sequence analysis of OnCD96

The ORF of *OnCD96* was 1371 bp, encoding a protein of 456 amino acids with a signal peptide, the nucleotides and deduced amino acid sequence are shown in Fig. 1A. The physico-chemical properties of OnCD96 were analysed using the ExPASy-protParamtool and the results showed that the molecular weight was 49.8 kDa and the theoretical isoelectric point was 5.67. Using TMHMM Server, the transmembrane domain was predicted. The results showed that there was a transmembrane domain composed of 23 amino acids (aa 384–406), indicating that the OnCD96 is a type I transmembrane glycoprotein. The structure domain analysis results are represented by a topological graph (Fig. 1B), there were two Ig-like domains in the extracellular domain. While the cytoplasmic region was only 50 amino acids long, it contained two important molecular motifs: a proline-rich motif (aa 423–427), and a 6-aa immunoreceptor tyrosine-based inhibitory motif (ITIM).

By comparison with other species, we found that OnCD96 shared the highest identity with *Maylandia zebra* (63%), but low homology with human and mouse. It is worth noting that the proline-rich motif and ITIM motif (S/V/I/LxYxxI/V/L) are very conservative in these species (Fig. 2). The evolutionary tree depicting the relationship between Nile tilapia CD96 and other species CD96 is shown in Fig. 3. The results showed that Nile tilapia and *Maylandia zebra* are clustered into one branch, thus have a closer relationship.

3.2. Subcellular localization of OnCD96

The subcellular localization of OnCD96 was determined by pDsRed-

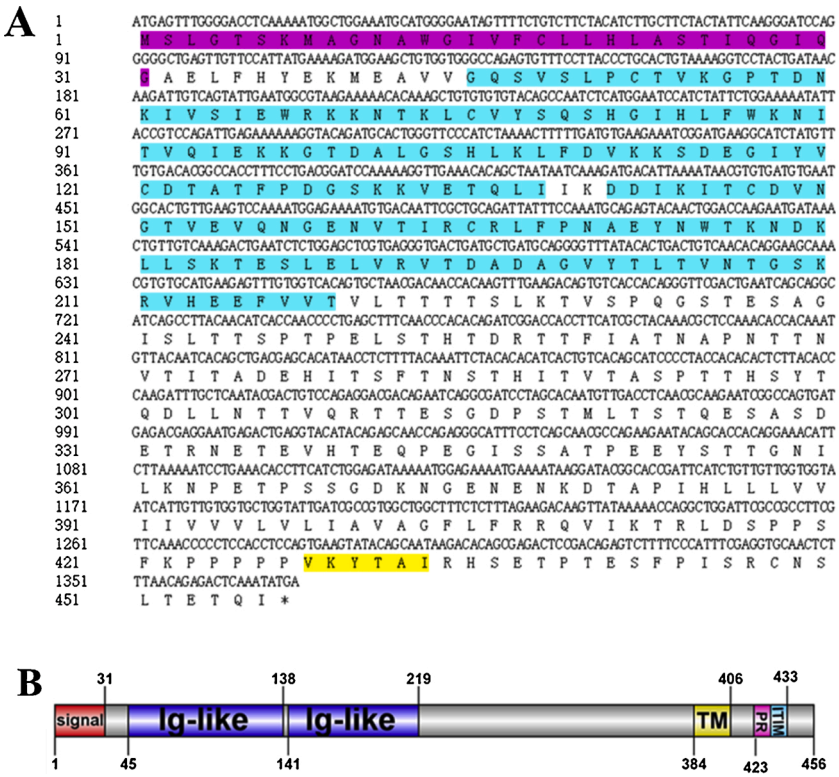

Fig. 1. Sequences and domain topology of OnCD96. (A) The OnCD96 nucleotide and deduced amino acid sequence. The signal peptide is represented in purple, the two Ig-like domains (aa 45-138,141-219) in blue-gray, the ITIM motif in yellow. (B) The domain topology of OnCD96.

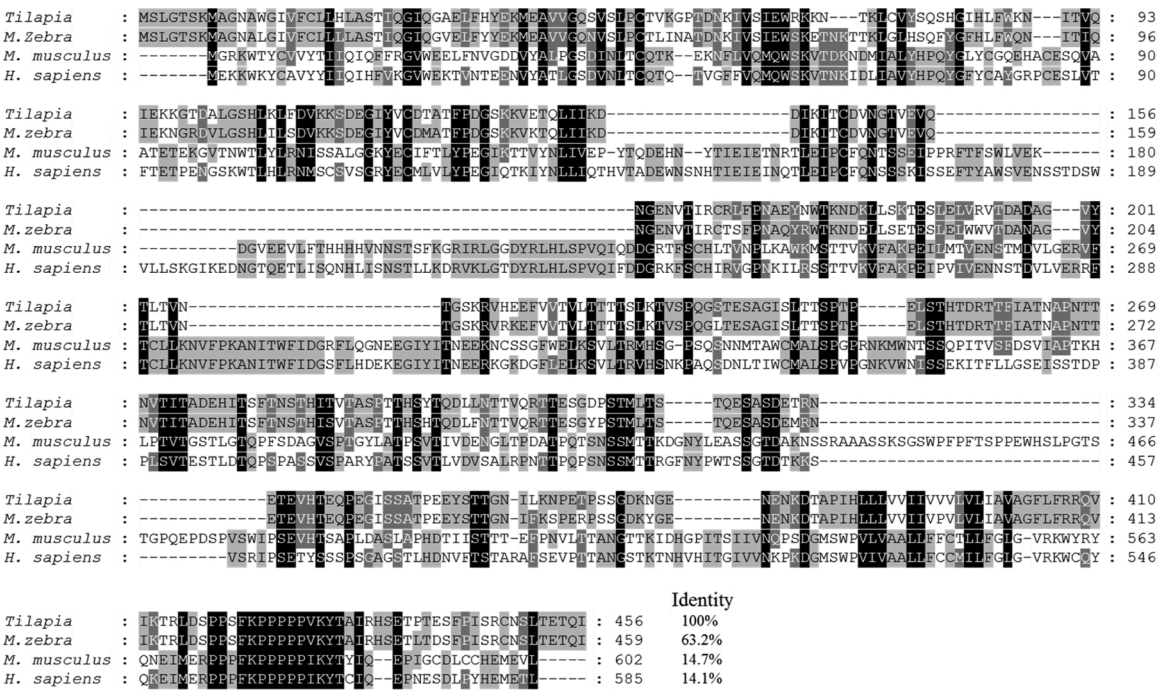

Fig. 2. OnCD96 amino acid multiple sequence alignment with other known homologs. The GenBank accession number of CD96 are as follows: *M. zebra* (XP_004550103.2), *M. Musculus* (NP_115854.2), *H.sapiens* (NP_937839.1).

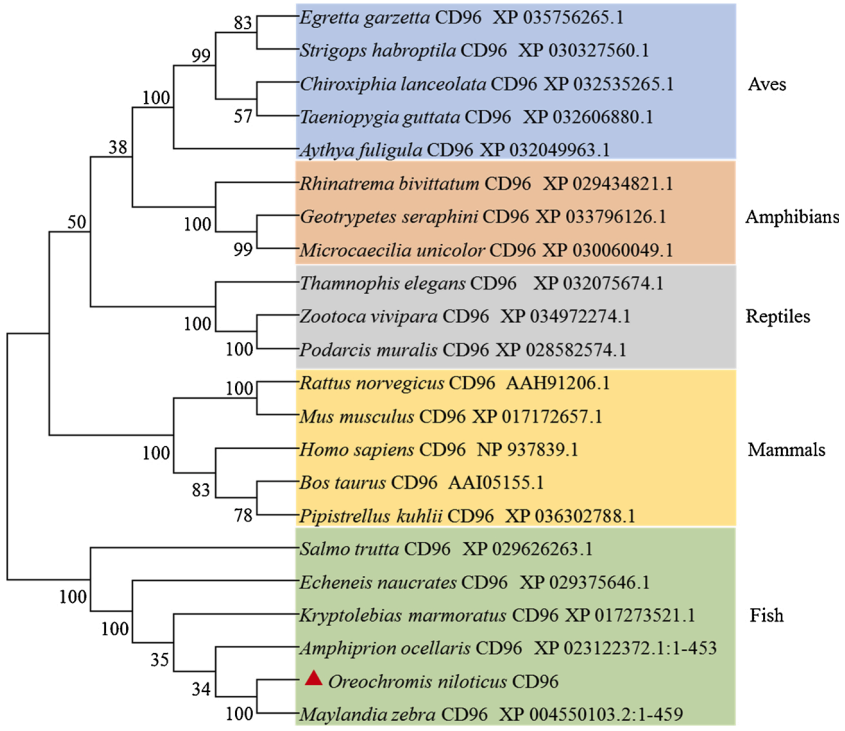

Fig. 3. Phylogenetic tree of OnCD96 among other species. Numbers on branches represent the bootstrap values on 1000 replicates. Phylogenic analysis was done by N-J method in MEGA6.0.

OnCD96 fusion protein expression levels in HEK-293 T cells (Fig. 4). The red fluorescence in pDsRed-OnCD96 fusion protein of HEK-293 T cell were both in cytoplasm and plasma membrane.

3.3. Tissues expression of OnCD96 mRNA

Quantitative fluorescent results showed that the *OnCD96* gene can be expressed in all tissues. Interestingly, *OnCD96* was expressed at relatively higher levels in the liver, while low expressions were observed in the other tissues examined (Fig. 5).

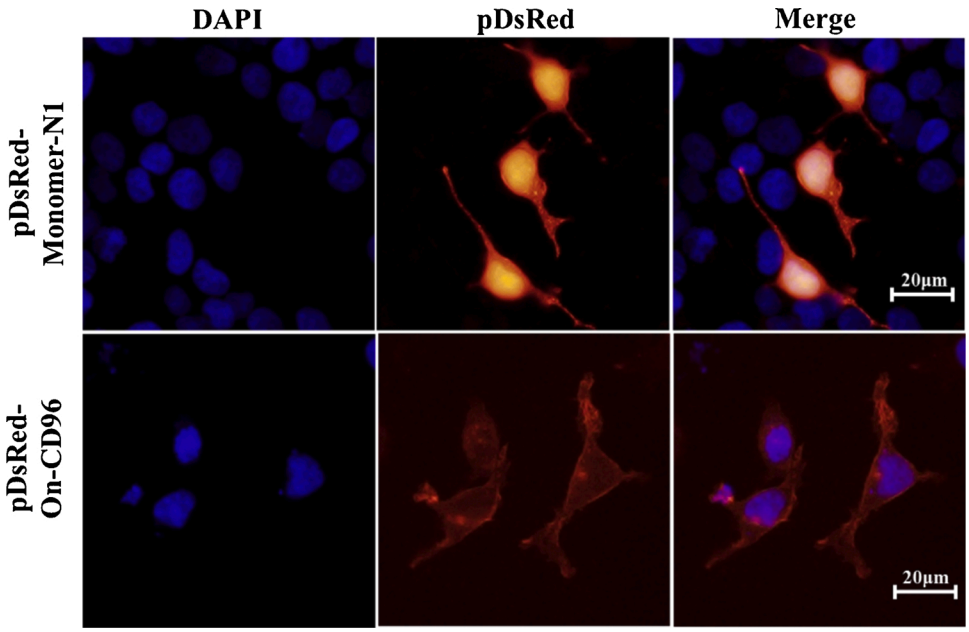

Fig. 4. Subcellular localization of OnCD96 in HEK-293 T cells. The cells were transfected with pDsRed-Monomer-N1 (upper row) or pDsRed-OnCD96 (lower row) and the nucleus was stained with 4′,6- dimaidino-2-pheny-lindole (DAPI).

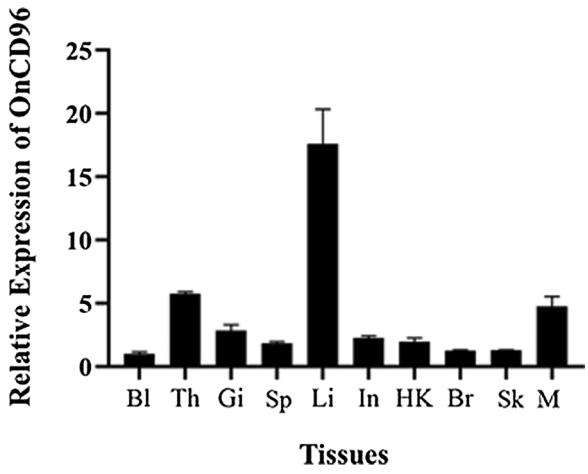

Fig. 5. *OnCD96* expression in healthy fish tissues. The values are shown as mean ± S.D.
Note: Bl (Blood), Th (Thymus), Gi (Gill), Sp (Spleen), Li (Liver), In (Intestine), HK (Head-kidney), Br (Brain), sk(Skin), m (muscle).

3.4. Temporal expression of OnCD96 in different tissues after stimulation by S. agalactiae and poly I:C

We determined *OnCD96* expression in different tissues under different stimulations to confirm whether OnCD96 was involved in immune response. The results showed that *OnCD96* was up-regulated initially and then decreased after the stimulation. In detail, the expression of *OnCD96* in the thymus, brain, and spleen reached their peaks at 72 h, 48 h, and 24 h, respectively after *S. agalactiae* stimulation (Fig. 6A–C). Whereas the expression of *OnCD96* in the thymus and spleen reached their peaks at 6 h and 24 h, respectively after poly I:C stimulation but the expression in the brain had double peaks, one at 24 h and the other at 96 h (Fig. 6D–F).

3.5. Kinetics of OnCD96 expression over a TD antigen response

The results showed that the peak of *OnCD96* expression in blood appeared at 28 d for the first challenge and appeared at 21 d for the second challenge, while in head-kidney both peaked at 14 d. Taking a holistic viewpoint, OnCD96 expressions were up-regulated in the immune tissues during the first and second challenge. Moreover, the time of significant up-regulation in the second challenge was earlier than the first challenge in the blood (D 1 vs. D 21 p.i.) (Fig. 7A), and head kidney (D 7 vs. D 14 p.i.) (Fig. 7B). Although the up-regulation in the spleen was statistically insignificant, it still has certain reference significance (D 1 vs. D 14 p.i.) (Fig. 7C).

3.6. Interaction of OnCD96 with OnNecl5

The interaction between OnCD96 and OnNecl5 proteins was proved by the colors of the hybridized yeasts. The results were shown in Fig. 8. For autoactivation groups, yeasts containing pGADT7-OnCD96/pGBKT7 and pGBKT7-OnNecl5/pGADT7 could grow on corresponding SD medium, the colonies were white, but no colonies could grow on SD medium with Aureobasidin A, suggesting that OnCD96 and OnNecl5 themselves had no transcriptional activity. In the positive control group, yeasts containing pGBKT7-53/pGADT7-T1 grew on double dropout, triple dropout, and quadruple dropout medium and the colonies were blue. In the negative control group, yeasts containing pGBKT7-Lam/pGADT7-T grew on double dropout medium and the colonies were white but unable to grow on the triple dropout and quadruple dropout medium. In the experimental group, yeasts containing pGADT7-OnCD96/pGBKT7-OnNecl5 grew on double dropout, triple dropout, and quadruple dropout medium and the colonies were blue, indicating that there might be interaction between OnCD96 and OnNecl5.

4. Discussion

In this study, we successfully cloned the CD96 with a 1371 bp ORF from Nile tilapia. Sequence analysis revealed that OnCD96 shared 14%-63% identity with other species, which is consistent with the results of previously studied immune related molecules such as CD28 (Bernard et al., 2006), CD48 (Wang et al., 2020), and CD80/CD86 (Huang et al., 2018). In the evolution of vertebrates, they must undergo strict selective evolution to adapt to the environmental changes from aquatic to terrestrial and to fight more pathogens. The rapid evolution of genes

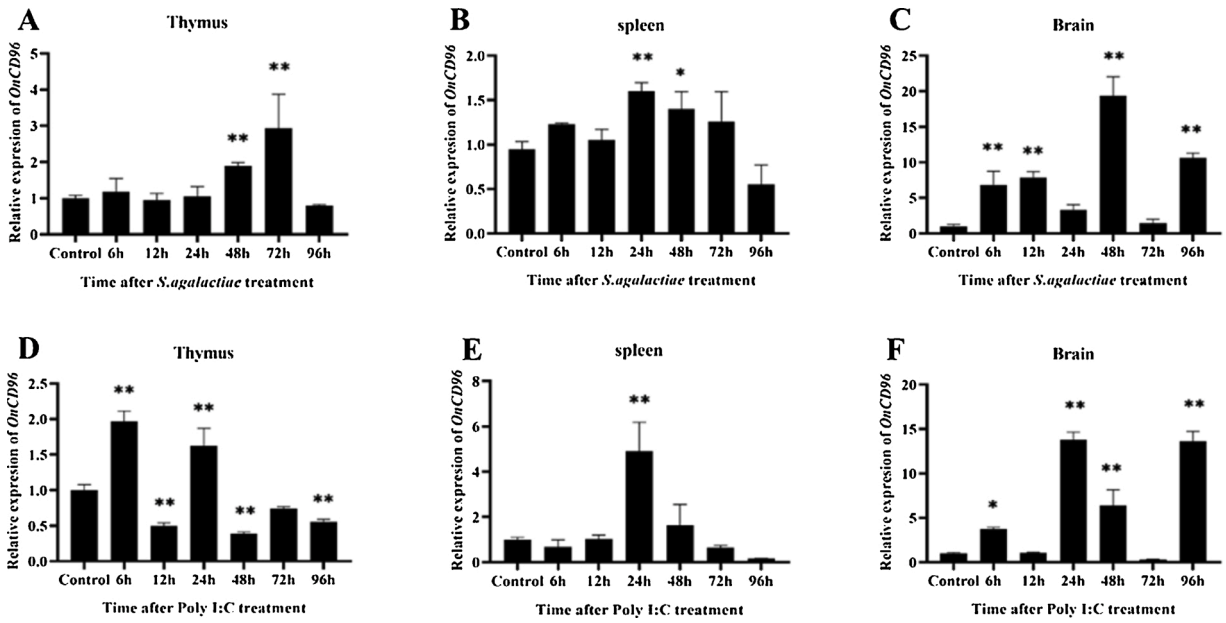

Fig. 6. Temporal expression of *OnCD96* in immune tissues after infection with *S. agalactiae* (A–C) and poly I:C (D–F). The values are shown as means ± S.D. Significant difference was indicated by asterisk, *p < 0.05, **p < 0.01.

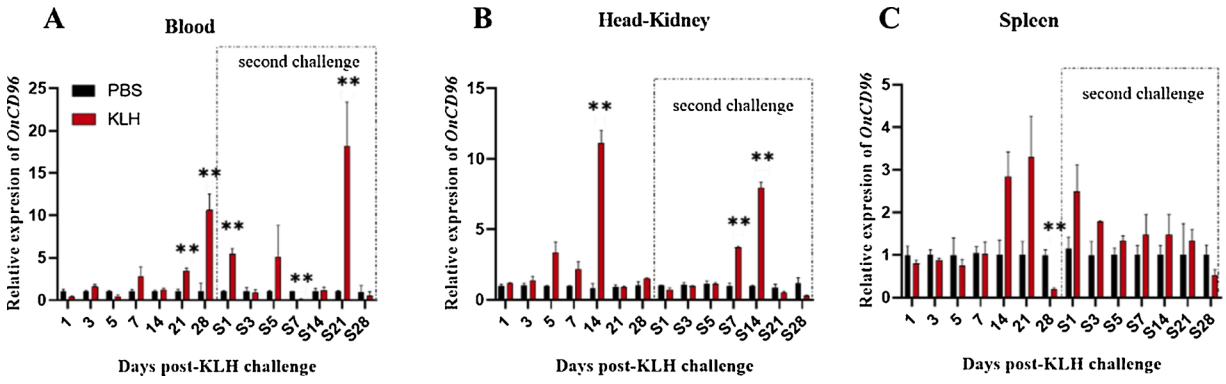

Fig. 7. Temporal expression of *OnCD96* in immune tissues after KLH challenge. The values are shown as means ± S.D. Significant difference was indicated by asterisk, *p < 0.05, **p < 0.01.

associated with T-cell immunity poses a challenge to the study of T cell immunity in fish (Gan et al., 2016a). These evolutionary pressures may lead to less conserved sequences of T cell immune-related genes among different species. Structurally, we found that OnCD96 has only two Ig-like domains, as compared to three in human CD96 (hCD96) and mouse CD96 (mCD96). The first domain of CD96 has been demonstrated to harbor the epitopes essentially required for Necl5 binding and the magnitude of binding is modulated by the second domain in the case of hCD96 but not mCD96 (Meyer et al., 2009). Further research about the binding site of OnCD96 to the ligand is needed. The cytoplasmic domain of CD96 is short but consisted of several important motifs: ITIM and proline-rich motifs. In the immune system, the receptors with one or more ITIMs are considered as negative regulators (Unkeless and Jie, 1997) and when these receptors bind to their corresponding ligands, they can cause Src family protein kinase activation. Under the action of Src family protein kinases, the tyrosine in the receptor ITIM is phosphorylated, which can recruit protein tyrosine phosphatase (PTP) and exert an inhibitory effect on the activation signal transmission (Otipoby et al., 2001; Muraille et al., 2000). This can reduce the excessive activation of T cells and has an important role in reducing excessive inflammation. In general, the sequence of OnCD96 shared low homology with other species, but the important functional domains were completely conserved, indicating that OnCD96 may have a function similar to other CD96s.

In healthy tilapia, *OnCD96* was expressed in all tested tissues, suggesting a constitutive and ubiquitous expression of OnCD96. With low expression in most of the immune organs, we speculate that the expression level of *OnCD96* also has an important relationship with the T cell status. CD96 is mainly expressed in T cells, but at low levels on resting T cell (Gramatzki et al., 1999), therefore, OnCD96 was lowly expressed in most of the tissues of healthy fish. Interestingly, the highest expression of OnCD96 was observed in the liver. Given that the liver in a fish serves several functions including digestion, waste removal, and storage, and always in complex physiological activities, it is, therefore, understandable that *OnCD96* expression in the liver was relatively higher.

In recent years, diseases triggered by *S. agalactiae* and Tilapia lake virus (TiLV) have caused huge economic losses to the tilapia farming industry (Ferguson and Beltran, 2014; Eyngor et al., 2014; Evans et al., 2009). For the study of the role of OnCD96 in antibacterial and antiviral activities, we have tested the *OnCD96* mRNA expression after injection with *S. agalactiae* (one kind of Gram-positive bacteria) and poly I:C (one kind of viral analogues). The results showed that *OnCD96* expressions were significantly up-regulated in the thymus, brain, and spleen in a

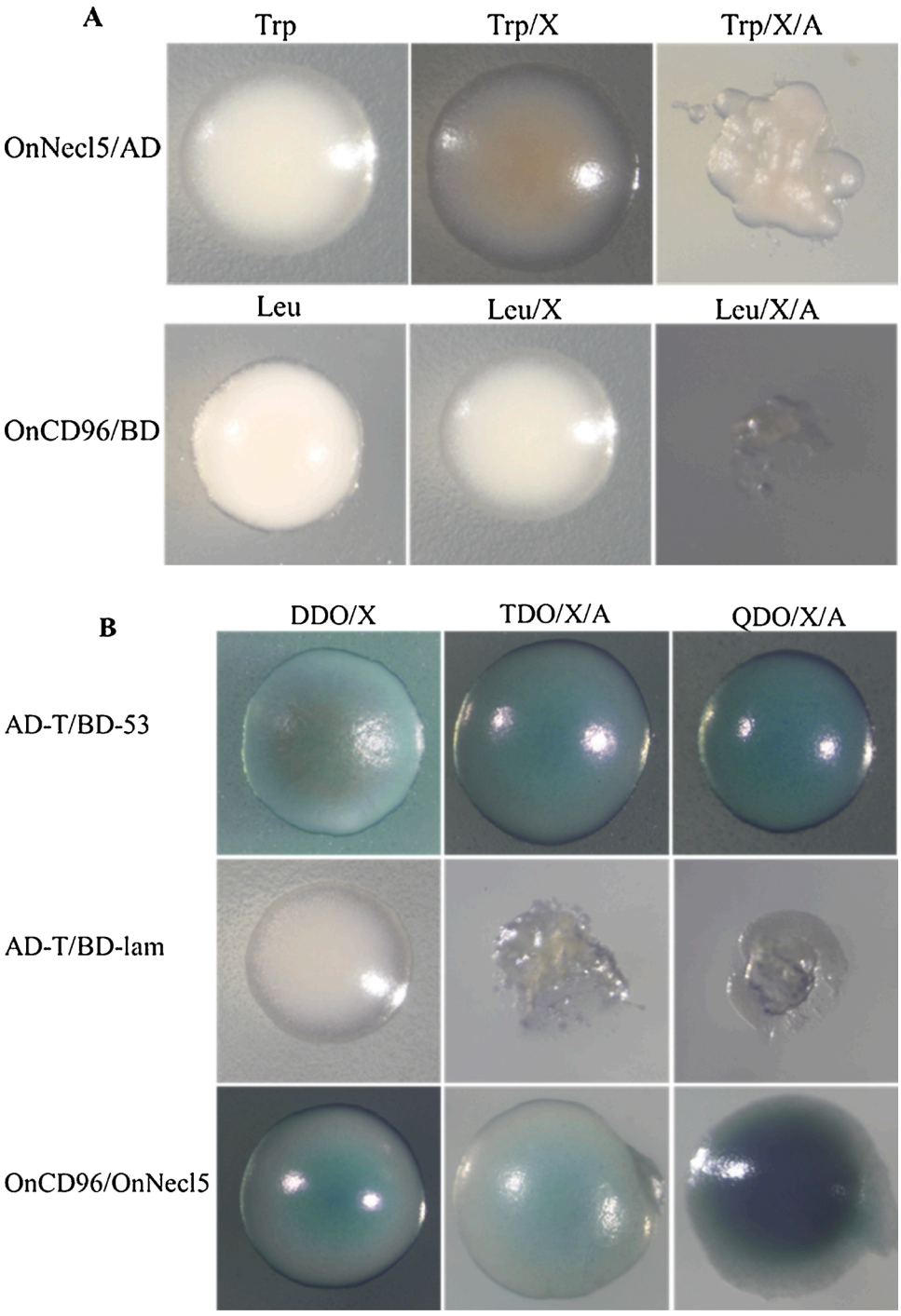

Fig. 8. The results of Yeast two-hybrid assay. SD/–Leu refers to medium with all essential amino acids except for leucine, SD/–Trp refers to medium with all essential amino acids except for tryptophan, SD/–Leu/–Trp represents Double Dropout(DDO), SD/–His/–Leu/–Trp represents Triple Dropout(TDO), SD/–Ade /–His/–Leu/–Trp represents Quadruple Dropout (QDO), X is X-a-Gal, A is Aureobasidin A. (A) Identification of self-activation of the plasmids of yeast two-hybrid system. (B) Identify protein interaction between OnCD96 and OnNecl5 by yeast two-hybrid system. pGADT7-OnCD96/pGBKT7-OnNecl5 represents the experimental group, PGADT7-T/PGBKT7-53 represents the positive control, PGADT7-T/PGBKT7-Lam represents the negative control.

time-dependent expression pattern. Previous studies have shown that the brain, as the most important central nervous system of an animal, is usually difficult to be infected by bacteria due to the presence of the blood-brain barrier (Robinson and Meyer, 1966). However, researches have shown that *S. agalactiae* can break through this barrier, infect tilapia brains, and cause severe meningitis (Schuchat, 1998). The CD96 molecule in mammals may function at a time after T and NK cells have penetrated the endothelium using integrins and selectins, when they are actively engaging diseased cells and moving within areas of inflammation (Hristo et al., 2018). Therefore, we speculate that the CD96 molecule is actively involved in the brain inflammation of tilapia caused by *S. agalactiae* and poly I:C. It is worth noting that in the brain, *OnCD96* displayed a second expression peak in the later period after poly I:C infection, probably due to the delay in the T cell-mediated acquired immunity. A large number of T cells are distributed in the thymus and spleen (Cesta, 2006), when the pathogen invaded, the increased expression of OnCD96 activated the T cells and participated in resisting pathogen infection. The significantly high expression of *OnCD96* was mainly after 24 h, suggesting that *OnCD96* might affect the T cell function at those later stages of the immune reaction which is consistent with hCD96 (Wang et al., 1992). In summary, OnCD96 may play an important role in the tilapia immune response against *S. agalactiae* and viruses.

Studies have shown that the production of antibodies during the immune response of fish also has thymus-dependent and independent pathways similar to mammals. Among them, the production of thymus-

dependent antibodies requires the assistance of T cells. Since KLH is used as a TD antigen (Ryota et al., 2013), we stimulated the healthy tilapia by KLH and then tested the dynamic trends of *OnCD96* expression. The results showed that *OnCD96* significantly increased in the blood and head-kidney after KLH stimulation, and compared with the first immunization, it showed a sequential expression pattern in the second immunization with faster expression, higher intensity and longer duration. These findings are similar to human CD96 (Dougall et al., 2017). It is worth noting that the peaks of *OnCD96* were always at later stages after stimulation which may be related to the expression characteristics of the CD96 molecule itself, because CD96 in mammals has been confirmed to participate in adhesive interactions of activated T and NK cells during the late phase of the immune response. More detailed mapping within human T cells showed that CD96 expression was highest on $CD4^+$ and $CD8^+$ T cells with an effector memory phenotype, transcriptional profiling revealed that $CD96^{high}$-expressing memory T cells had unique resting and activated transcription landscapes, and $CD96^{high}$ T cells also had a distinct functional phenotype following activation (Chiang et al., 2020). The significant up-regulation of OnCD96 was earlier in the second challenge than the first challenge, suggesting a positive correlation between OnCD96 expression level and memory response. However, there are some differences with mammals, such as that the peak of the second immunization is not always higher than the first, perhaps the immune memory of fish is different from that of mammal, but this remains unclear and further study is required. In addition, it could be seen that the expression intensity of OnCD96 on peripheral blood and head-kidney were higher than spleen, similar to tilapia CD6 and CD226 (Unpublished data), which may be due to the difference in the distribution characteristics of fish T lymphocytes. It has been established in fish that T cells are very abundant in the thymus, and are also present in the main lymphoid tissues, the head-kidney and the spleen, but the exact distribution of T cells remains to be precisely described (Nakanishi et al., 2015). Based on the above results, we inferred the OnCD96 may participate in the host's defense against T cell-dependent pathogen infection.

The subcellular localization results showed that OnCD96 existed in both the cytoplasm and plasma membrane of HEK293 T, since HEK293 T is a mammalian cell line, so the exact distribution of OnCD96 in tilapia tissues or cells needs to be further studied by preparing monoclonal antibodies, using immunofluorescence or in situ hybridization. The interaction of CD96 and Necl5 has been demonstrated in mammals (Muraille et al., 2000) and the yeast two-hybrid technique used in this study also proved their interaction. However, the yeast two-hybrid method alone is not enough to fully prove that CD96 and Necl5 can interact, because yeast two-hybrid verifies that membrane-protein interactions have limitations and require specific modifications (Mehla et al., 2017), it is necessary to use pull down and Co-IP methods for further verification. The demonstration of the interaction between CD96 and Necl5, and the functional study of the interaction in T cell activation will be the focus of our future work.

In summary, we successfully cloned CD96 from tilapia which was found in all the tissues examined. Subcellular localization studies showed that OnCD96 was distributed both in cytoplasm and plasma membrane. CD96 can respond to *S. agalactiae* and poly I:C and is involved in the T cell-dependent response in Nile tilapia. We also found the interaction between OnCD96 and OnNecl5 through yeast two-hybrid assay. These findings will lay a strong foundation for the further study of OnCD96 in tilapia.

Declaration of Competing Interest

The authors report no declarations of interest.

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