Fish and Shellfish Immunology 88 (2019) 36–46

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Purification and functional characterization of serum transferrin from Nile tilapia (*Oreochromis niloticus*)

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ARTICLE INFO

*Keywords:*
*Oreochromis niloticus*
Transferrin
Iron binding
Antibacterial activity
Inflammation

ABSTRACT

Transferrin (TF), an iron-binding multifunctional protein, could participate in the iron-withholding strategy, an effective antimicrobial defense mechanism in innate immunity, and is involved in host defense against pathogenic infection. In this study, a TF homologue (OnTF) was purified from serum of Nile tilapia (*Oreochromis niloticus*) through a two-step affinity chromatography, and characterized its antibacterial function and the role in inflammatory response. The identification by mass spectrometry showed that peptide sequence of the purified OnTF was highly consistent with its amino acids sequence, containing two conserved iron binding lobes: N-lobe and C-lobe. The native OnTF was able to bond iron ions, and possessed capability to inhibit the growth of both bacterial pathogens (*Streptococcus agalactiae* and *Aeromonas hydrophila*) *in vitro*. Upon infections of *S. agalactiae* and *A. hydrophila*, the expression of OnTF protein was significantly up-regulated *in vivo* and *in vitro*. In addition, the OnTF participated in the regulation of inflammation, migration, and enhancement of phagocytosis and respiratory burst activity in head kidney macrophages/monocytes. Taken together, the results of this study indicated that OnTF is likely to involve in innate immunity to play a role in host defense against bacterial infection in Nile tilapia.

## 1. Introduction

Iron is an indispensable trace element in organisms and plays an important role in many physiological activities, including oxygen transport, electron transport, DNA synthesis, etc [1–3]. However, excess iron not only stimulates the production of harmful free radicals in organisms, but also facilitates the proliferation of pathogenic microorganisms *in vivo* [1,2]. Therefore, maintaining an appropriate balance of iron for the health in the organism is important. Iron homeostasis is maintained through a series of protein interactions and regulatory mechanisms, in which transferrin (TF) is important in iron storage and transport [3–5]. It not only participates in the transport and metabolism of iron, and regulates the balance of iron ions, but also regulates the inflammation, respiration, cell proliferation, differentiation and participate in the immune system, and has a function of antimicrobial and bactericidal [6–8]. Therefore, TF is widely existed in organisms from invertebrates to vertebrates with comprehensive physiological function.

In vertebrates, TF is mainly synthesized in the liver, and also expressed in blood, skin and kidney [5,9,10]. The TF protein is composed with two homologous globular domains, the N-lobe and the C-lobe linked by a hinge region. Each domain has a conserved amino acid sequence that can bind with ferric iron molecule [11]. The N-terminal has stronger combination ability for $Fe^{3+}$ binding, while the C-terminal is more important for binding with transferrin receptor (TfR) [12]. According to the tissue source of secretion, TF is divided into serum transferrin, lactoferrin, ovotransferrin and melanotransferrin [3,13]. Among them, serum transferrin is also known as serotransferrin, which is first synthesized by hepatocytes and circulated in the blood [3,10]. As the most-studied member of the transferrin family, serotransferrin plays a crucial role in regulating dynamic equilibrium of iron ions, and contributing in immune response and the host defense [10,14,15].

At the present time, transferrin had already been reported in variety of teleost, which includes the gene clone of the *TF* from Atlantic cod (*Gadus morhua*) [16], rainbow trout (*Oncorhynchus mykiss*) [17], goldfish (*Carassius auratus*) [18], common carp (*Cyprinus carpio*) [19], channel catfish (*Ictalurus punctatus*) [3] and Wuchang bream

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https://doi.org/10.1016/j.fsi.2019.02.060
Received 12 December 2018; Received in revised form 22 February 2019; Accepted 25 February 2019
Available online 01 March 2019
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(*Megalobrama amblycephala*) [5], and the purification of TF from goldfish [6] and common carp [20]. These studies mainly focused on the cloning, expression, evolution and polymorphism analysis, and the role in antibacterial and immune response. However, until now, the role of TF in non-specific cell immunity in host defense against pathogenic infection remains unknown in teleost. Our recent article has shown the identification of *OnTF* from Nile tilapia (*Oreochromis niloticus*) and represented the expression and function in binding bacterial pathogens [10]. Here we purified and identified natural serotransferrin protein of Nile tilapia, investigated its binding activity of iron ions and capability to inhibit two pathogenic bacterial growth *in vitro*, examined its expression upon bacterial challenges *in vivo* and *in vitro*, and explored its function in non-specific cell immunity including inflammation, migration, phagocytosis and killing. These conclusions demonstrated that OnTF is essential in resistant to the bacterial infection in Nile tilapia.

## 2. Animals, samples and methods

### 2.1. Animals feeding and samples collection

The Nile tilapia weighing 200 ± 20 g (for cell separation), 500 ± 50 g (for serum collection) were acquired and cultured as previous publication [10]. The New Zealand white rabbit and Balb/c female mice were feeding by Guangdong Medical Laboratory Animal Center, and raised in the Zoology Laboratory of South China Normal University. The healthy rabbit at an age of 18 weeks and healthy mice at an age of 8 weeks were suitable for the preparation of polyclonal antibody. The animal feeding agreement was authorized by the University Animal Care and Use Committee of the South China Normal University.

Prior to venous blood collection, tilapia were anaesthetized with MS-222 (Aladdin, USA). The peripheral blood of Nile tilapia was collected and processed by heparinized sterile syringe refer to references [21,22].

### 2.2. The preparation of rabbit anti-(r)OnTF polyclonal antibody

The recombinant Nile tilapia transferrin (r)OnTF was prepared following the previous study [10], the immunological methods were according to the publications [23,24]. New Zealand female white rabbits were vaccinated subcutaneously (five locations) with 500 μg of (r)OnTF and emulsified with complete Freund's adjuvant (FCA) (Sigma, USA) at 2 week intervals. The following immunization was performed three times with incomplete Freund's adjuvant (FIA), the tail venous blood was gathered prior to immunization and 3 day after individual immunization. The positive hybridization reaction was analyzed by western-blot and antibody titer was detected by ELISA [25]. The polyclonal antibody reached a titer against (r)OnTF with 2,200,000 units/mL. The rabbit was anaesthetized again for collecting a large amount of antiserum and storaged at −20 °C.

The purification of polyclonal antibody was performed using Melon Gel Monoclonal IgG Purification Kit (Thermo, USA), the steps were simplified as the protocols. The rabbit antiserum was dialyzed with 1 × Melon™ Gel Purification Buffer (pH 6.5–6.7), then added to the Melon Gel in the gravity column and rotated at room temperature for 5 min. The gravity column was placed in 50 mL centrifuge tube for centrifugation, 3000 × *g*, 4 °C for 1 min. Then collecting the supernatant and detecting the protein concentration by NanoDrop 2000.

### 2.3. Isolation of OnTF and preparation of mouse-against OnTF polyclonal antibody

The rabbit-against-(r)OnTF polyclonal antibody was combined to the CNBr activate sepharose-4B beads (GE Healthcare, USA) as described by Cuatrecasas [26]. The conjugate antibody was 15 mg per milliliter beads and each gravity column was added 1 mL prepared beads. The Nile tilapia serum was centrifuged at 500 × *g*, 4 °C for 15 min and diluted with same amount of sterile PBS, adding 5 mL diluted tilapia serum to each column and rotated overnight at 4 °C. The uncombined fractions was washed with a 10-fold column volume PBS solution and the binding protein was eluted with 2 mL 50 mM glycine buffer (pH 8.0) for three times. These eluted fractions were further dialyzed for 4 times at 4 °C against PBS and concentrated by PEG 20,000 to a total volume of 2 mL. The concentrated supernatant was loaded to Con A-Sepharose 4B beads (GE Healthcare, USA) and rotated overnight at 4 °C. The bound proteins were eluted with PBS with the addition of 20 mM Tris-HCl and 0.2 M methyl α-D-mannopyranoside [27]. It was validated that transferrin did not bind to the column with the inhabitation of methyl α-D-mannopyranoside, therefore the unbound fractions were accumulated. The SDS-PAGE and Western-blot were used to investigate the purification of protein and the positive protein band was cut down for the analysis of mass spectrometry (BPRC, China). The OnTF protein was digested with trypsin, and the peptides were examined by LC-MS/MS using the Q Exactive™ HF hybrid quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher, USA). The specific way was briefly described as follows. The flow rate was 600 nL/min, and the mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). The elution gradient used was 6%–95% mobile phase B for 52 min. The spray voltage was 2.2 KV and the caplillary temperature was 320 °C, the normalized collision energy was 50%. The primary quality range of collection is 300–2000 *m/z*, and the secondary scanning range is 100–1400 *m/z*. The search engine was Mascot, the first-level error of database was 20 ppm and the error of the second-level was 0.5 Da, the peptide coverage analysis was according to the protein number of GI 89475215.

The eluted OnTF was wielded as antigen to immune 8 weeks female Balb/c mice. The detail steps were refer to the previous description [10]. The polyclonal antibody confirmed to be 700,000 units/mL, then the antiserum was gathered and stored at −80 °C.

**Table 1**
Primers in this study.

| Primers | Nucleotide Sequence (5′-3′) | Purpose |
|---|---|---|
| *β*-actin-F | CGAGAGGGAAATCGTGCGTGACA | Control, RT-qPCR |
| *β*-actin-R | AGGAAGGAAGGCTGGAAGAGGGC | Control, RT-qPCR |
| qIL-6-F | ACAGAGGAGGCGGAGATG | RT-qPCR |
| qIL-6-R | GCAGTGCTTCGGGATAGAG | RT-qPCR |
| qIL-8-F | GATAAGCAACAGAATCATTGTCAGC | RT-qPCR |
| qIL-8-R | CCTCGCAGTGGGAGTTGG | RT-qPCR |
| qMIF-F | CACATCAACCCTGACCAAAT | RT-qPCR |
| qMIF-R | GCCTGTTGGCAGCACC | RT-qPCR |

### 2.4. Iron-binding capacity

The detection of OnTF binding with iron was according to the method of Nynca [28]. The OnTF iron release was accomplished by consecutive dialysis against 0.1 M citrate/acetate buffer (pH 4.3), water, 0.1 M perchloric acid buffer, water, 0.1 M Tris-HCl (pH 8.0). The function of binding with iron was measured by continuous adding of ferric chelate complex Fe (III)-nitrilotriacetate (Fe-NTA) to desaturate OnTF. Fe-NTA was available by $FeCl_3$ and NTA solution in a molar ratio of 1:2 (pH 6.0), then detected the absorbance at 465 nm for dilution adjustment.

### 2.5. Antibacterial assay for OnTF

Antimicrobial activity of OnTF was evaluated by antimicrobial assays following the previous method [8]. *S. agalactiae* or *A. hydrophila* was grown in broth at 30 °C for 6 h with shaking at 180 rpm. The bacteria was centrifuged at 5000 × *g* for 10 min, the precipitation was dissolved in corresponding medium and adjusted the absorbance at

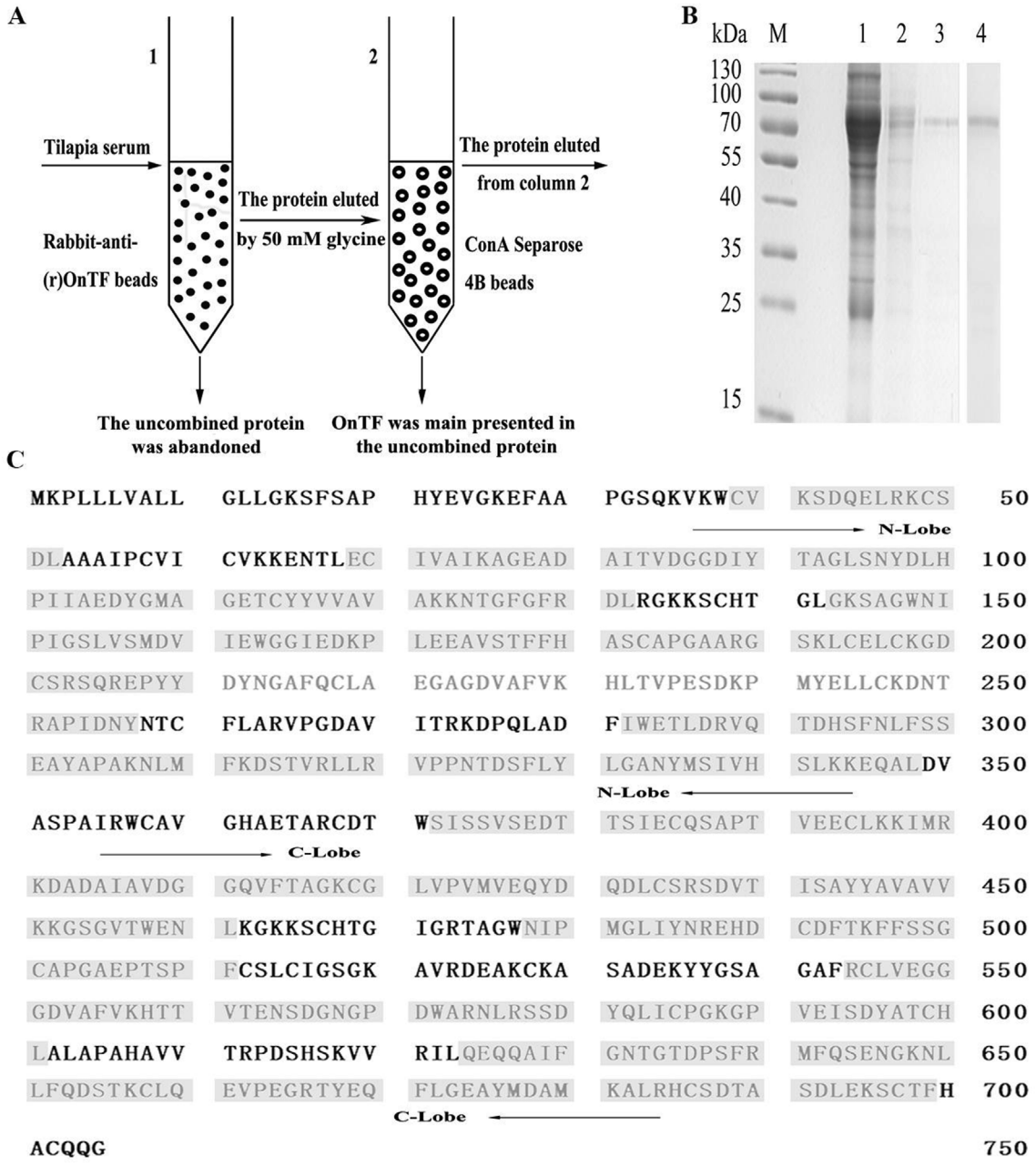


**Fig. 1.** Purification of tilapia serum transferrin (OnTF) (A), validation of polyclonal antibody for (r)OnTF (B) and mass spectrometry coverage of TF sequence (C). Lane M, markers; Lane 1, the tilapia serum; Lane 2, the purified protein after affinity (anti-(r)OnTF) column (bound fractions); Lane 3, ConA column elution of unbound fraction; and Lane 4, western blot analysis of OnTF (B). Peptides identified by mass spectrometry are shaded in grey and the predicted N-Lobe and C-Lobe are indicated by arrows (C).

O.D. 600 to 0.6. Then, 100 μL bacteria solution, $FeCl_3$ (final concentration of 1 μM) and OnTF (final concentrations of 50 and 250 μg/mL, all the protein used in the following study was endotoxin treatment) were added to 5 mL medium in sterile centrifuge tubes. The mixture was settled at 30 °C and measured the absorbance at O.D. 600 hourly to quantify the cell densities. At the end of the last measurement, the bacteria in the centrifuge tubes were diluted to an appropriate concentration, and 100 μL diluted bacteria cells was used to coat plates. In order to ensure the creditability of the data, the assays were performed in triplicate.

### 2.6. ELISA to determine OnTF concentration

A competitive-inhibition ELISA was used to detect the OnTF concentration in Nile tilapia serum and macrophages/monocytes culture supernatant after the stimulations of *S. agalactiae* and *A. hydrophylia*, the serum and culture supernatant were collected and store at −80 °C [10]. The 96-well plates (Corning, USA) were coated with purified OnTF 10 μg/mL at 4 °C overnight. The plates were then blocked with 0.5% BSA-TTBS for 2 h at 37 °C. The stimulated serum (25-fold dilution), culture supernatant (5-fold dilution) and mouse anti-OnTF pAb (1:3200, determined previously) were placed in each well and incubated for 1 h at 37 °C. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Southern Biotech, USA). Then, measured the O.D. 405 with a microplate reader (Thermo, USA). The negative and positive controls were also included on the relevant plate, and the results were calculated according to the standard curves pre-made. The concentration gradient of OnTF used in

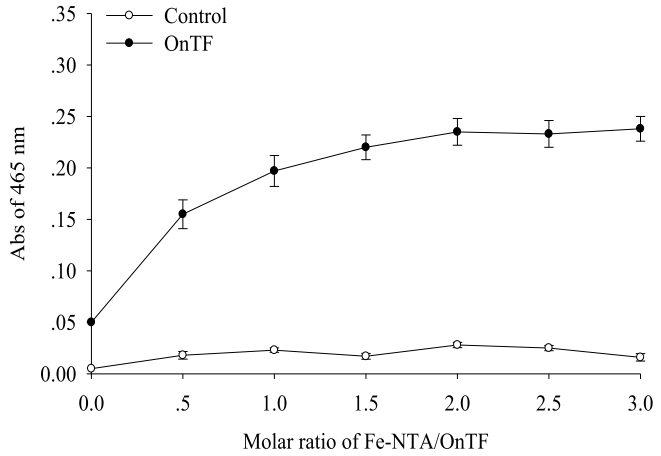


**Fig. 2.** Spectrophotometric titration of purified OnTF with Fe-NTA. Samples of tilapia serum apo-TF (iron-free TF; 1.6 mg/mL in 0.1 M Tris-HCl with 0.005 M NaHCO3, pH 8.0) were added with aliquots of 5 mM ferric chelate complex Fe-NTA and read at 465 nm. The error bars represent standard deviation (SD) (n = 3) and significant difference was indicated by different letters.

the preparation of the standard curve was 1 mg/mL, 800 μg/mL, 600 μg/mL, 400 μg/mL, 200 μg/mL, 100 μg/mL. And then the protein concentration was 2-fold diluted from 100 μg/mL. After 13 dilutions, the minimum concentration was nearly 0.01 μg/mL. Unless special instructions, the plates were washed three times between each two adjacent steps with 1 × TTBS (0.1% Tween 20, pH 8.0).

### 2.7. Isolation and culture of head kidney macrophages/monocytes

The separation of head kidney macrophages/monocytes was according to the former papers with some modifications [10,29]. The Nile tilapia head kidneys were washed and cut into pieces and then make into cell suspensions. Then the cell suspensions were loaded onto a separation liquid consisting with 10 mL of 54% percoll (Sigma, USA) blanketed with 10 mL of 31% percoll. After centrifugation, the macrophage/monocyte fraction of the 31%–54% interface was collected. The cells were washed with L-15 medium, the quantity and viability was appraised by 0.4% trypan blue exclusion, and the concentration of the cells was $2 \times 10^7$ cells/mL with 1% penicillin/streptomycin and 10% FBS in the culture medium and incubated in 96-well microplates (Corning, USA) (100 μL/well) overnight. After incubation, the non-adherent cells were removed and re-suspended the adherent cells in L-15 medium, with 10% FBS and 1% penicillin/streptomycin. The final concentration of the cells was diluted to $1 \times 10^6$ cells/mL.

The RNA and cDNA template synthesized for the study in quantitative real-time PCR were prepared according to reference [10].

### 2.8. Expression analysis of IL-6, IL-8 and MIF after OnTF stimulation

The relative expressions of Nile tilapia *IL-6*, *IL-8* and *MIF* mRNA in macrophages/monocytes after the stimulation of OnTF were performed on the 7500 Real Time PCR System (Life Technologies, USA). The treatment group was challenged with OnTF (10 μg/mL, 50 μg/mL, 250 μg/mL, 1.25 mg/mL), and the PBS treated group was set as control. The primers used were shown in Table 1. The PCR reaction volume and program was set as before [10]. The relative expression of each gene was calculated using tilapia *β*-actin gene expression, and the results were further determined by means of the $2^{-\Delta\Delta Ct}$ method [30].

### 2.9. Assay for effect of OnTF on phagocytosis

The OnTF affecting the phagocytosis was detected by flow cytometry according to the previous methods [29,31]. The macrophages/monocytes were prepared as in section 2.7, and the FITC labeled *S. agalactiae* and *A. hydrophila* were doing as before [29]. The labeled bacteria suspensions (200 μL) were mixed with 100 μL of OnTF (250 μg/mL), then added the $FeCl_3$ solution to a final molar concentration of 2 μM and incubated for 60 min avoid light. Then centrifuged and re-suspended the bacterial cells with 300 μL PBS and 300 μL cell suspension ($2 \times 10^7$ cells/mL), and incubated for 60 min with shaking. The control group was treated with PBS (pH 7.4). Then the cell suspensions were centrifuged with over 3% BSA in PBS supplemented at 100 × *g*, 4 °C for 10 min to eliminate the non-ingested bacteria. The extra fluorescence was quenched by mixing 1 μL trypan blue (0.4%) [31]. The samples were analyzed in FACS Aria III (BD, USA) flow cytometer with a 488 nm argon-ion laser, and 10,000 events were collected for each sample.

### 2.10. Assay for effect of OnTF on respiratory burst

To explore the effect of OnTF on respiratory burst, flow cytometric analysis was performed the previous steps [32]. In the positive group, 300 μL prepared monocytes/macrophages cell suspension ($2 \times 10^7$ cells/mL) and 100 μL of OnTF (250 μg/mL) were incubated at 37 °C for an hour. The control group was substituted by PBS (pH 7.4) processed similarly. A total of 10,000 events were collected for each sample.

### 2.11. Statistical analysis

The experiments in present study were repeated three times for statistical analyses. The data analyzed using one-way ANOVA were represented as mean ± standard deviation (SD) by SPSS 17.0 software, statistical significance was indicated by different letters. The figures were made by Sigma Plot 10.0 software.

## 3. Results

### 3.1. OnTF purification and mouse-against OnTF polyclonal antibody detection

The protein was analyzed by SDS-PAGE electrophoresis and western-blot. The prepared mouse polyclonal antibody was the primary antibody for the western-blotting analysis. In the electrophoresis gel, a slice including OnTF was excised for identification, it indicated that a band (~75 kDa) corresponding to the OnTF molecular mass could be detected, the polyclonal antibody had a significant positive reaction with the band (Fig. 1B). The mass spectrometry analysis showed that the amino acid sequence of this protein was consistent with that of OnTF, the amino acid sequence coverage of the peptide reached more than 70% (Fig. 1C). There were two representative structure domains, and approximately 310–330 amino acids in each lobe (N-lobe C-lobe) (Fig. 1C).

### 3.2. Iron-binding capacity of OnTF

In order to detect the iron-binding capacity of Nile tilapia transferrin, the denatured purified OnTF was mixed with Fe-NTA and read the absorbance at 465 nm. The titration of OnTF with Fe-NTA showed that 100% of saturation of OnTF was achieved at a molar ratio of 2 mol of $Fe^{3+}$: 1 mol of OnTF (Fig. 2), and there was no significant change in titration curve in PBS control group.

### 3.3. Iron-dependent inhibition of bacterial growth

The antibacterial activity of OnTF affirmed the iron depriving ability in the present study. Here, we analyzed the growth and propagation of two iron-dependent bacteria, *S. agalactiae* and *A. hydrophila*. It showed a significant antibacterial effect against both bacteria in a dose

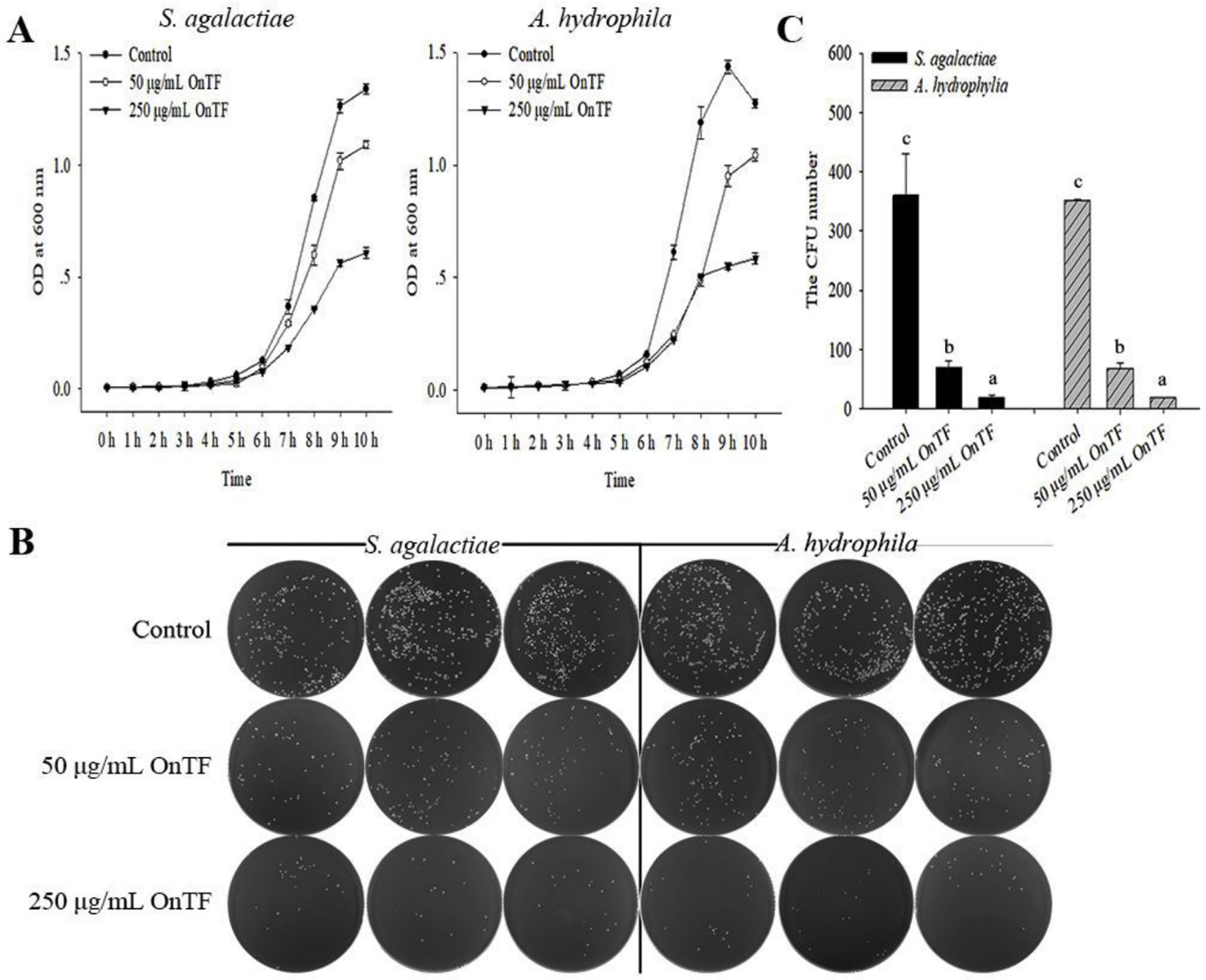


**Fig. 3.** Iron-dependent bacterial growth inhibition. The bacteriostatic activity of OnTF. (A). The growth curves of *S. agalactiae* and *A. hydrophila*, X-axis indicates time, and Y-axis indicates the O.D. at 600 nm. Each point in the graph represents the mean of at least three assays. The bacterial liquid in (A) at time of 10 h was diluted to an appropriate concentration, and then 100 μL was used to coat plates (B). The colony-forming number of each plate was analyzed in (C). The error bars represent standard deviation (SD) (n = 3) and significant difference was indicated by different letters.

dependent manner (Fig. 3). This result affirms that the transferrin was an acute-phase protein with the function of inhibiting bacterial proliferation by sequestering $Fe^{3+}$ ion from the surrounding.

### 3.4. Dynamics of OnTF expression by stimulation in vivo and in vitro

In order to investigate the effect of *S. agalactiae* and *A. hydrophila* on the OnTF expression in tilapia serum and macrophage/monocyte culture supernatant, an ELISA assay was adopted to measure OnTF concentrations. As shown in Fig. 4A, upon challenged with *S. agalactiae* and *A. hydrophila*, the OnTF concentration was significantly up-regulated in serum at 12–168 h post-stimulation, with the optimal expression nearly 3500 μg/mL at the time range of 48–96 h. In addition, the dynamic change of OnTF concentration in culture media showed a similar trend after *in vitro* stimulation of *S. agalactiae* and *A. hydrophila*, and the maximum concentration reached the peak at time of 48 h with a value of 173.3 μg/mL and 240.5 μg/mL, respectively (Fig. 4B).

### 3.5. Effect of OnTF on inflammatory and migration reaction

The isolated head kidney macrophages/monocytes were challenged with a gradient concentration of OnTF (10 μg/mL, 50 μg/mL, 250 μg/mL, 1.25 mg/mL) and the PBS challenged was set as control (Fig. 5). After challenged with OnTF, the expression of *IL-6* was significantly up-regulated by all four concentrations. When the OnTF concentration increased from 10 μg/mL to 250 μg/mL, the significant increase of *IL-6* expression raised 16-fold–33.3-fold at 48 h p.i. (Fig. 5A). However, stimulated with the 1.25 mg/mL OnTF, the expression trend of *IL-6* was only with a 16-fold increase at time of 48 h (Figs. 5A–4).

The expressions of *IL-8* in all stimulation groups were also significantly up-regulated. Challenged with a low concentration of OnTF (10 μg/mL), the expression of *IL-8* reached the peak rapidly, with an increase of 9-fold (Fig. 5B). With the increase of OnTF concentration, the expression of *IL-8* did significantly raised compared with the control, but only maintaining a 5-fold increase (Fig. 5B). The expression pattern of *MIF* mRNA was similar to that of *IL-6* by OnTF stimulation, with a significant up-regulation (2.6 fold) (Fig. 5C) after treated with the 250 μg/mL OnTF.

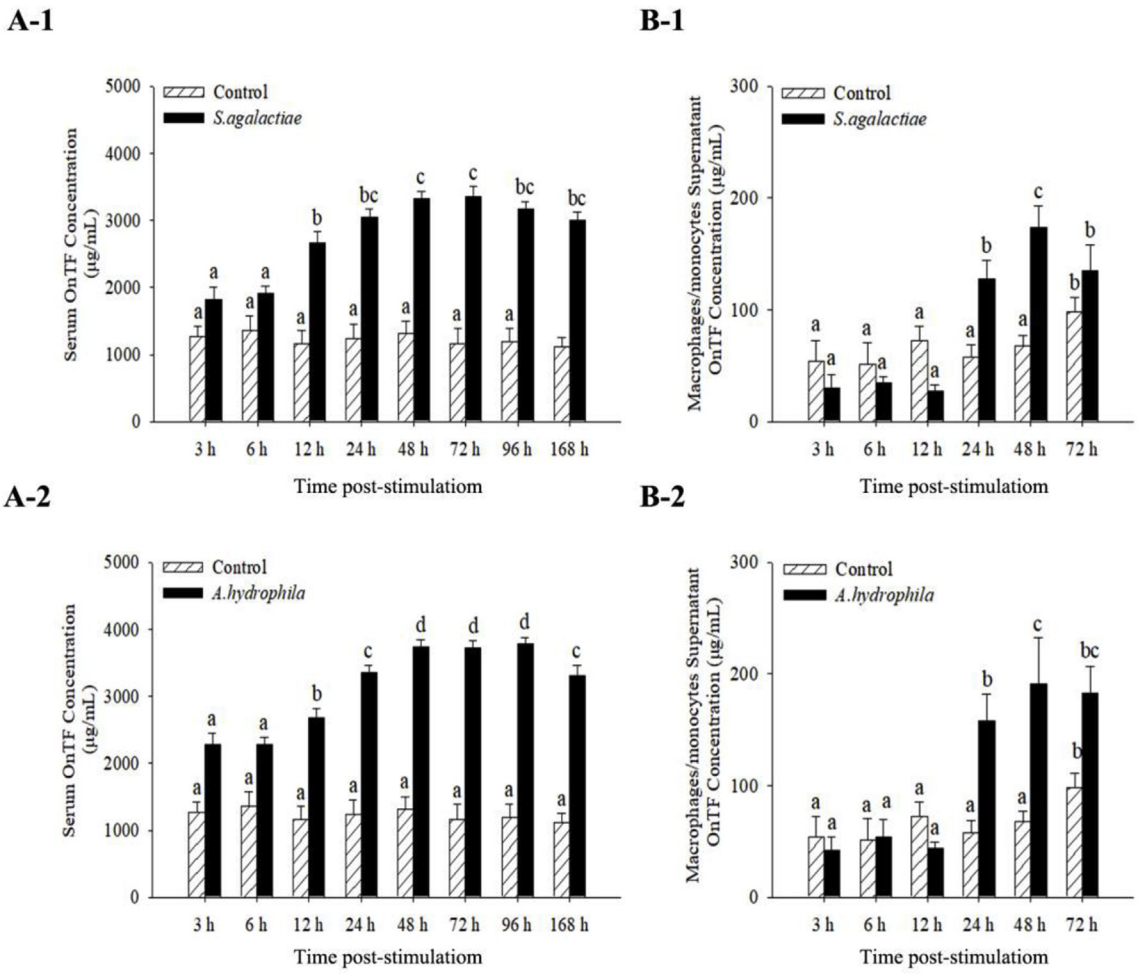


**Fig. 4.** The dynamic change of OnTF concentration in serum and cell supernatant after *S. agalactiae*, *A. hydrophila* stimulation and the PBS control. The error bars represented standard deviation (SD) (n = 3) and significant difference was indicated by different letters.

### 3.6. OnTF promotes the phagocytosis

The OnTF promotes the phagocytosis of macrophages/monocytes was determined by flow cytometer. As demonstrated in Fig. 6, the phagocytosis rates of the macrophages/monocytes were determined by phagocytosis the OnTF-treated bacteria (250 μg/mL, the concentration determined according to the above experiment). The fluorescence was limited in a gate to ensure the data analysis. According to the statistics, the OnTF-treated *S. agalactiae* and *A. hydrophila* were more conducive to the phagocytosis rates of the macrophages/monocytes, which differed from that of non-treated bacteria (Fig. 6C). All of the above indicated that the OnTF protein could regulate the phagocytosis of bacteria by macrophages/monocytes.

### 3.7. The regulation of OnTF to respiratory burst

With the aim of investigating the effect of OnTF on respiratory burst, the macrophages/monocytes were treated with a proper concentration of OnTF (250 μg/mL) prior to PMA stimulation. The results were determined by flow cytometer and the fluorescence intensity of macrophages/monocytes were significantly higher after the OnTF treatment than those of the control group (Fig. 7). It showed that the OnTF protein could promote the respiratory burst of macrophages/monocytes.

## 4. Discussion

Transferrin, as a multifunctional iron-binding protein, not only plays an important role in transporting iron ions and iron metabolism in the body, but also participates in cell respiration, cell growth, proliferation and regulation of immune system [6,7,33]. In addition, it possesses antibacterial function, which plays an important role in the innate immune defense against bacterial infection [8]. In the current study, the native transferrin was isolated and purified from Nile tilapia serum by a simple and effective method, which possessed apparent iron binding and bactericidal ability to bacterial pathogens, and participated in regulating inflammation, migration, phagocytosis and enhancement of respiratory burst. These results demonstrated that OnTF might be involved in Nile tilapia host defense against bacterial infection.

Transferrin, a non-heme β-globulin with iron binding activity, mainly exists in plasma and extracellular fluid and is an indispensable component in body fluids [34]. TF regulates iron metabolism, prevents free iron from producing toxic and side effects on body cells by free radical formation, which plays a transport role in iron absorption, storage and utilization [33]. TF protein contains two domains, N-Lobe and C-Lobe, which conformation is maintained by 19 pairs of intrachain disulfide bonds, with 8 pairs of N-terminal and 11 pairs of C-terminal [34,35]. Disulfide bonds can not only stabilize the internal structure of secondary and tertiary peptide chains and mediate the formation of quaternary structure between peptide chains, but also are essential in the conformation of proteins as well as the binding of iron ions and TF receptor action [36,37]. When TF binds to metal ions, its

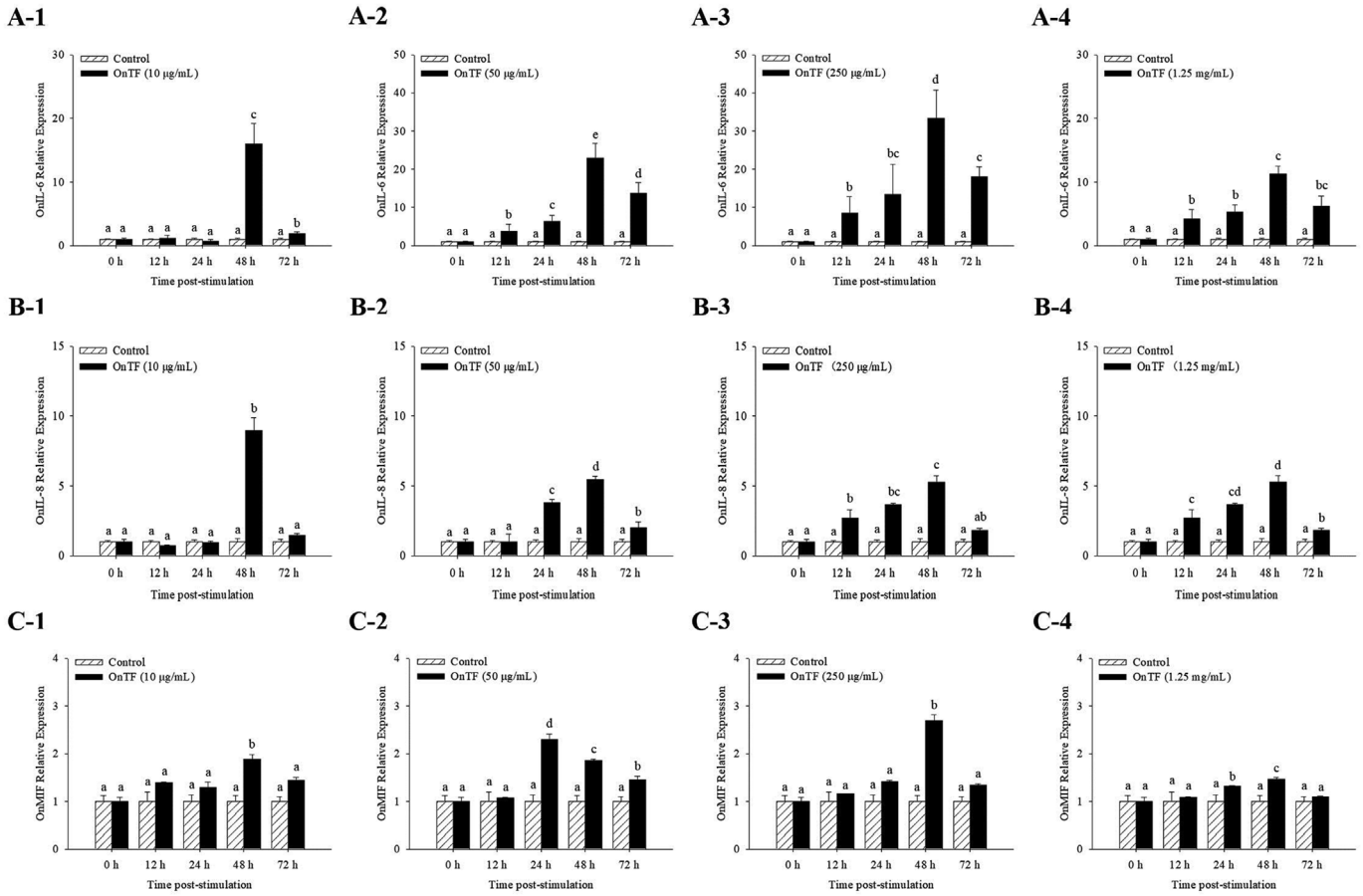


**Fig. 5.** Time response of interleukin-6 (IL-6), interleukin-8 (IL-8) and migration inhibitory factor (MIF) gene expressions in macrophages/monocytes after challenge of different concentration OnTF. The isolation cells were incubated with medium containing 10 μg/mL, 50 μg/mL, 250 μg/mL and 1.25 mg/mL transferrin for 12–72 h. The cDNA was extracted from cells and subjected to qRT-PCR amplification. The mRNA level of different genes was normalized to that $\beta$-actin and fold units were calculated deciding the values of the PBS treated cells. The error bars represent standard deviation (SD) (n = 3) and significant difference was indicated by different letters.

conformation changes significantly and the structure becomes more compact and stable [38,39]. Each domain of TF contains four iron-binding sites and two anion-binding sites, which coordinate the transport of one $Fe^{3+}$ ion [10,40]. In this study, OnTF protein was proved to have an effective iron binding activity, since the PBS control was not capable of binding with Fe-NTA (Fig. 2). Previous studies have found that the two lobes were different in iron-binding ability, the C-lobe binding iron more tightly and releasing iron more slowly, and the N-lobe more important in maintaining the ferro-binding activity [9,15,40]. Moreover, the mammalian TF was verified to be a glycoprotein with two important N-glycosylation sites, which were active for iron transport [41,42]. However, these N-glycosylation sites seems to be lacking in the TF of Nile tilapia and other Cyprinidae fishes [10,18,20].

Iron as an important metal element is essential for living organisms, and innate immunity of the body effectively restricts iron availability to microbial invaders [43]. There are three ways for bacterial pathogens to absorb iron, including ingesting heme, releasing low molecular weight iron-containing cells, binding iron-containing TF through transferrin receptors (TfR) [44]. TF is an important factor that inhibits bacterial reproduction because of its anti-bacterial, bactericidal and self-protective properties [8,45,46]. In this study, the antimicrobial function of the OnTF was confirmed via the iron-dependent bacterial growth inhibition assay, in which the inhibition of bacterial growth was dose-dependent. The control group without the stimulation of OnTF could not inhibit the growth of bacteria. When compared with the control group, the group with transferrin addition was more effective in the inhibition of bacterial growth with the increase of OnTF concentration. This finding was similar to the previous study on rock bream (*Oplegnathus fasciatus*) [8]. Pathogens can promote iron to maintain at a high level, and then aggravate the effect of bacterial infection [43]. By competing with microorganisms for iron ions needed for their life activities and having the ability to chelate iron, TF is able to inhibit the growth of aerobic microorganisms, thus playing a bacteriostatic role. This could be well understood by the obvious increase of OnTF concentration in tilapia serum after bacterial stimulation. In addition, the increase trend of OnTF concentration in the cell supernatant was similar with that in the serum, significant difference was only found at time of 72 h in the control group. We assume that the secreted OnTF could not be rapidly consumed, so it remained in the cell supernatant.

In fish innate immune system, the non-specific cellular defense is an internal part. It is associated with many types of leukocytes, including macrophages, monocytes, and granulocytes [29,47]. The macrophages/monocytes own the ability to initiate inflammation and recruit immune cells, which is essential in the non-specific cellular defense [29,47]. Besides, there are a variety of cytokines, including interleukin 6 (IL-6), interleukin 8 (IL-8, CXCL8) and macrophage migration inhibitory factor (MIF), which are secreted by macrophages/monocytes. These cytokines play important roles in the inflammation and leukocyte migration process of host defense [25,48–50]. A series studies in mammals have shown that TF is involved in the inflammation and chemotaxis response [49–52]. In the current study, the relative expressions of *IL-6, IL-8* and *MIF* were obviously increase after the stimulation of OnTF, which was similar to that in human [50,51]. The IL-6, IL-8 and MIF, as essential

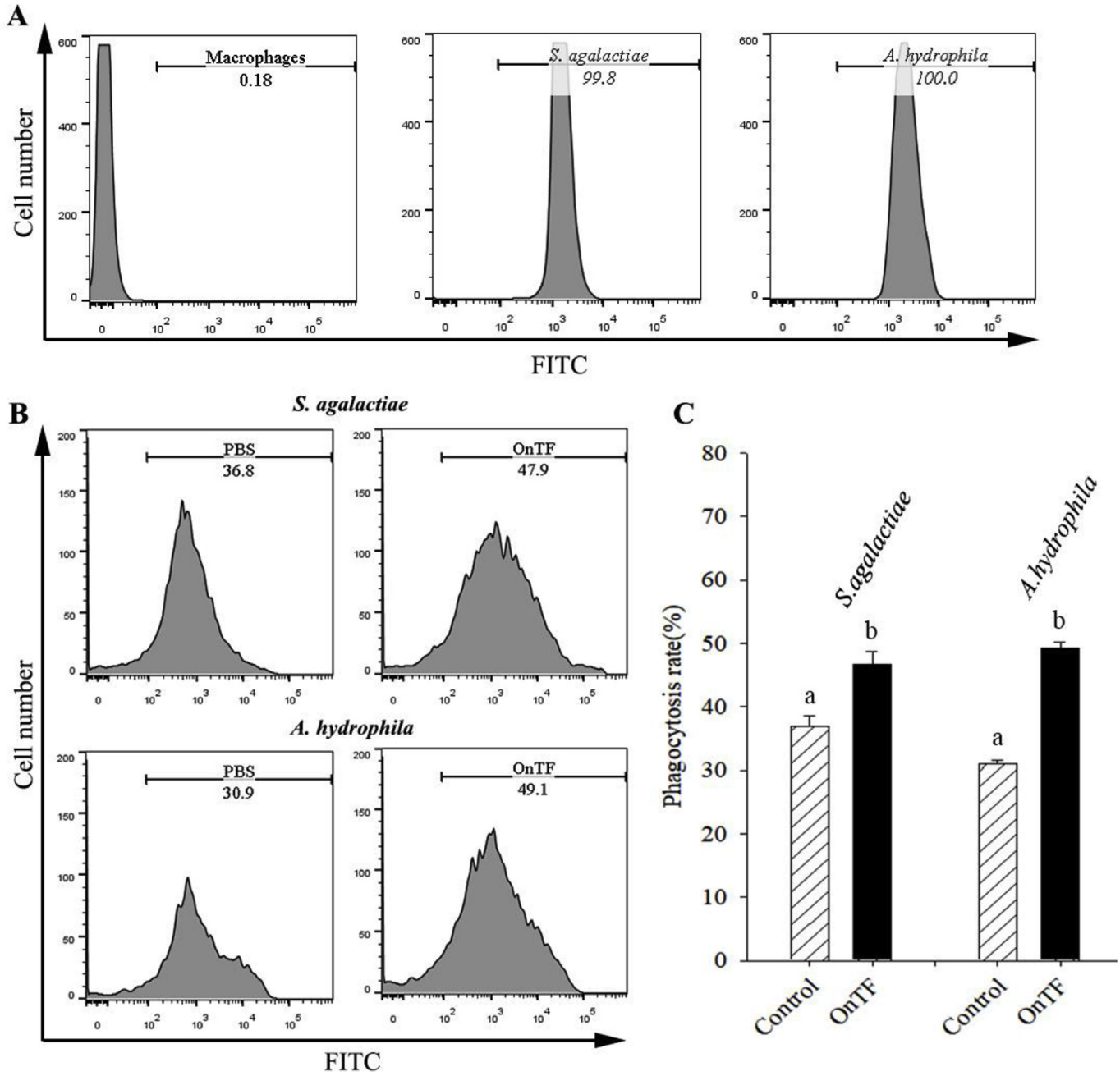


**Fig. 6.** Effect of OnTF on phagocytosis of Nile tilapia macrophages/monocytes. Flow cytometric analysis of the monocytes/macrophages phagocytosing *S. agalactiae* and *A. hydrophila*. Data show analyses of 10,000 events. The histogram of the cells alone, *S. agalactiae* alone and *A. hydrophila* alone (A). The histogram of flow cytometric analyses of the monocytes/macrophages phagocytosing *S. agalactiae* and *A. hydrophila* pre-incubated with PBS or OnTF (250 μg/mL) (B). The phagocytosis rates were shown near the marker. The results shown here were from one experiment out of three independent experiments. The histogram of the phagocytosis rates (C). The error bars represent standard deviation (SD) (n = 3) and significant difference was indicated by different letters.

cytokines, play important roles in the regulation of inflammation [53–58]. Besides, inflammatory cytokines may also cause tissue cells damage, and even cause degeneration and necrosis. Meanwhile, the body will take a series protective measures immediately, including making and release of anti-inflammatory factors, activating the feedback mechanisms to decrease the injury induced by excessive pro-inflammatory cytokines and hold the homeostasis and normal immune regulation [59,60]. As shown in Fig. 5, when the OnTF concentration raised to a certain high amount, the significant increases of *IL-6*, *IL-8* and *MIF* expressions were likely to be inhibited to reduce the damage caused by excessive inflammatory factors (Fig. 5). Collectively, the results imply that OnTF is probably involved in regulation of inflammatory response.

Phagocytosis, a basic defense mechanisms of organisms, participates in specific cells recognition, internalization, killing and digestion of invading microorganisms [47,61]. The involvement of macrophages/monocytes in non-specific immune defense can initiate phagocytosis, by clearing the pathogen through their specific receptors and interacting with pathogen-associated motifs on the surface of foreign particles [62]. The TF can interact with the specific receptors (TfR) of monocytes/macrophages to activate and enhance phagocytosis of pathogens [6,63]. In this study, the OnTF was demonstrated to be able to promote the phagocytosis of bacterial pathogens by Nile tilapia macrophages/monocytes, which may indicate the importance of TF in host defense

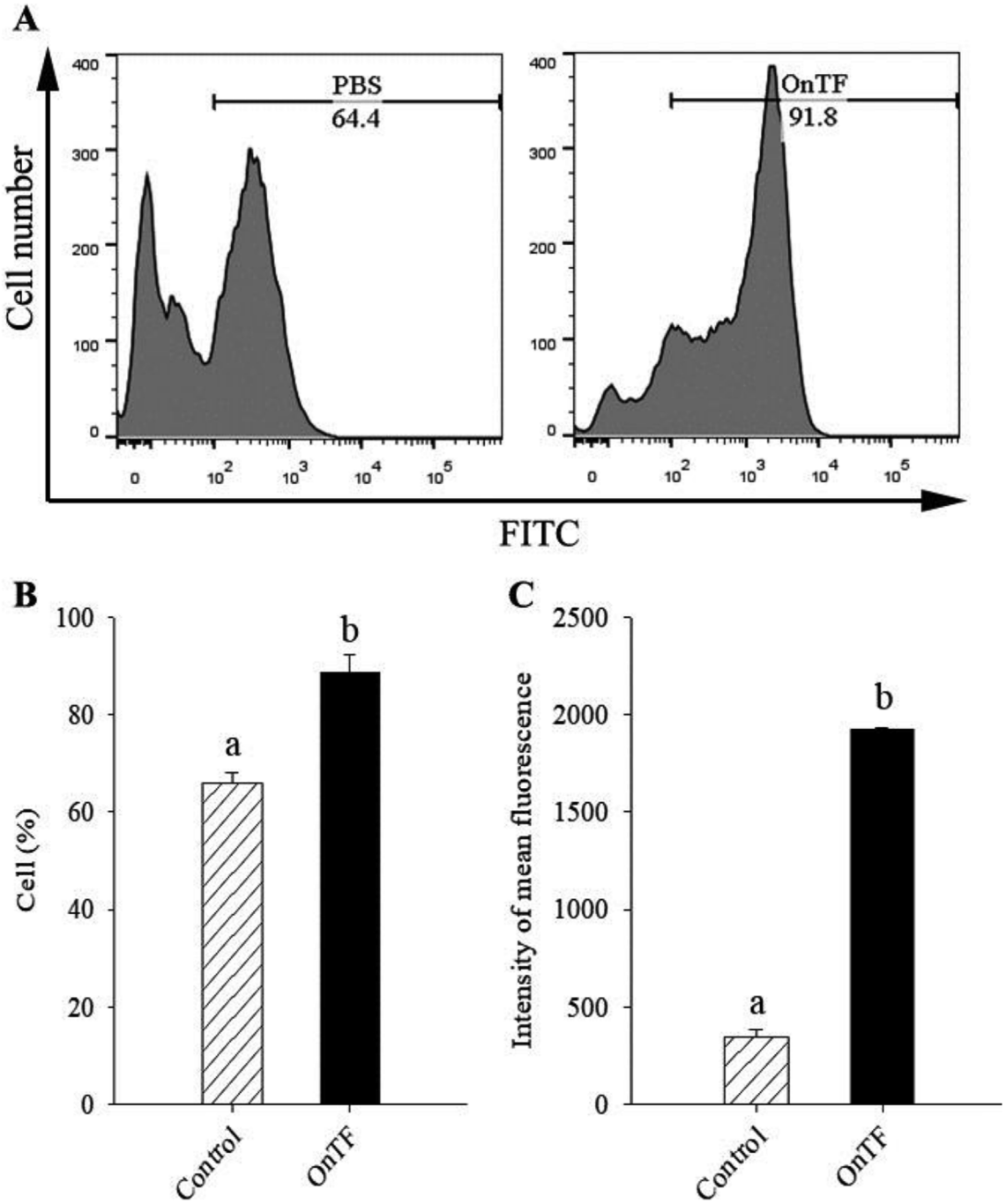


**Fig. 7.** Flow cytometric analysis of the head kidney monocytes/macrophages respiratory burst from Nile tilapia. The histogram of flow cytometric analysis of the monocytes/macrophages respiratory burst pre-incubated with PBS or OnTF (250 μg/mL) (A). The histogram of the positive cell rates (B). The histogram of the mean fluorescence intensity (C). The error bars represent standard deviation (SD) (n = 3) and significant difference was indicated by different letters.

against bacterial infection. The killing of pathogens by phagocytes can be classified into two groups: oxygen-dependent and oxygen-independent, and the respiratory burst is a major approach in which the phagocytes perform bactericidal function [47]. The phagocytes are activated and migrated to inflammatory sites when the pathogens invade. Then phagocytes devour and produce a series of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to eliminate pathogens [64–66]. The phagocyte respiratory burst optimizes the killing of pathogens through ROS and RNS, the nitric oxide (NO), superoxide ions ($O_2^-$) and a series of their protonated products [47,64]. In teleost, TF has been believed as a primary activator of macrophages, which can amplify the response of macrophages killing, such as goldfish and common carp [6,19]. In the current study, we found the OnTF protein was able to up-regulate the respiratory burst activity in macrophages/monocytes, indicating that OnTF may be involved in killing pathogens.

In summary, we successfully purified the natural transferrin protein from Nile tilapia serum and analyzed its multi-functions. After the stimulation of pathogens, the expressions of OnTF were increased significantly *in vitro* and *in vivo*. Further, the OnTF was likely to regulate the inflammation, migration, phagocytosis and respiratory burst activity in macrophages/monocytes. This work indicated that OnTF, as an important iron-binding protein, may possess antibacterial activity and take part in regulating the non-specific cellular immune defense, including inflammation, migration, phagocytosis and killing. Thus, the

OnTF is expected to involve in innate immunity and be essential in host defense against bacterial infection in Nile tilapia.

## Acknowledgement

This project was supported by National Natural Science Foundation of China (31472302, 31172432).

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