Fish and Shellfish Immunology 87 (2019) 265–274

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Functional characterization of a mannose-binding lectin (MBL) from Nile tilapia (*Oreochromis niloticus*) in non-specific cell immunity and apoptosis in monocytes/macrophages

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ARTICLE INFO

Keywords:
Oreochromis niloticus
MBL
Inflammation
Opsonization
Apoptosis
Monocytes/macrophages

ABSTRACT

Mannose-binding lectin (MBL), a soluble pattern recognition receptor, is able to recognize antigen and participate in non-specific cell immunity, such as regulation of inflammation, migration, opsonization, phagocytosis and killing, which plays an important role in innate immunity. In this study, we have investigated the contributing mechanisms and effects of MBL on the cell immunity of Nile tilapia (*Oreochromis niloticus*) monocytes/macrophages. The mRNA expression level of *OnMBL* was significantly up-regulated in monocytes/macrophages after *in vitro* bacterial infection (*Streptococcus agalactiae* and *Aeromonas hydrophila*). Recombinant OnMBL ((r)OnMBL) protein could participate in the regulation of inflammation, migration, and enhancement of phagocytosis and respiratory burst activity in monocytes/macrophages. Moreover, the (r)OnMBL could induce the apoptosis of monocytes/macrophages. Taken together, the results of this study indicated that OnMBL is likely to involve in immune regulation, which may play an important role in host defense of innate immunity in Nile tilapia.

1. Introduction

Pattern recognition receptors (PRRs) by the innate immune system plays an important role in the defense against infections that recognize pathogen associated molecular pattern (PAMP) on the surfaces of various pathogens such as bacteria, viruses, fungi and parasites [1,2]. It also plays an important regulatory role in maintaining the stability of the body environment and avoiding the occurrence of auto-reactive immune responses, and turns into a bridge connecting innate immunity and adaptive immunity [3,4]. C-type lectins (calcium-dependent) belong to a superfamily of PRRs that contain one or more C-type lectin like domains (CTLD) and recognize carbohydrate structures on the surface of pathogens and self-antigens [2,5]. The lectins are expressed in a variety of tissues and cell types, including natural killer cell, monocytes and macrophages [4,6]. By binding to the microbial surface, the lectins are able to decrease pathogen infection and participate in downstream immune regulation and homeostasis, such as cell proliferation, apoptosis, inflammatory reaction, chemotactic activity, and enhancement phagocytosis [4,6–9].

MBL is a key soluble PRR in the innate immune system and is a member of the C-type lectin superfamily [10]. MBL is a collagen-like complex protein and consists of multimers of an identical polypeptide chain of 32 kDa [10,11]. Each of the mature peptide chains is characterized by a carbohydrate-recognition domain (CRD), a hydrophobic neck region, a collagenous region (CLR) and a cysteine-rich N-terminal region. The MBL mainly uses the CRD recognition area and CLR effect area to play relevant functions [4,10–12]. In mammals, MBL is recognized to have a role in processes as diverse as recognition of altered self-structures, complement activation, opsonization, modulation of inflammation and apoptotic cell clearance [6,10,11]. Especially, MBL deficiency and MBL disease association studies have been a fruitful area of research and implicate an important role for MBL in infective, inflammatory and autoimmune disease processes [11,13].

In teleost, the MBL has been identified in many species, including the clone of the *MBL* gene from grass carp (*Ctenopharyngodon idella*) [14], Nile tilapia (*Oreochromis niloticus*) [12], zebrafish (*Danio rerio*) [15], common carp (*Cyprinus carpio*) [16], channel catfish (*Ictalurus punctatus*) [17], rainbow trout (*Oncorhynchus mykiss*) [18], goldfish (*Carassius auratus*) [19] and Japanese eels (*Anguilla japonica*) [20], and its purification from common carp [16], channel catfish [21] and

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https://doi.org/10.1016/j.fsi.2019.01.019
Received 16 October 2018; Received in revised form 6 January 2019; Accepted 11 January 2019
Available online 14 January 2019

rainbow trout [18]. These studies main examined the combining capacity and opsonization of the MBL, and its function in the lectin complement pathway of innate immunity. However, until now, whether teleost MBL plays a role in non-specific cell immunity and apoptosis is poorly understand. Our previous study has shown that MBL was identified from Nile tilapia (*Oreochromis niloticus*) and characterized at expression and agglutination functional levels [12]. Here we reported the multi-function study of the MBL from Nile tilapia in monocytes/macrophages. The mRNA expression level of *OnMBL* was investigated in monocytes/macrophages after *in vitro* bacterial infection (*Streptococcus agalactiae* and *Aeromonas hydrophila*). The assays for effect of recombinant OnMBL protein on non-specific cell immunity including inflammation, migration, phagocytosis and respiratory burst were demonstrated. Moreover, we aimed to investigate whether (r)OnMBL could influence the apoptosis of monocytes/macrophages and determine the molecular mechanisms underlying the interactions. These findings indicated that OnMBL is likely to play an important role in host defense of innate immunity in Nile tilapia.

2. Materials and methods

2.1. Fish

Nile tilapia (*O. niloticus*), about 500 ± 10 g, were obtained from Guangdong Tilapia Breeding Farm (Guangzhou, China), which were used for the isolation of head kidney monocytes/macrophages. Fishes were maintained in an automatic filtering and recirculating water system at 28 ± 2 °C for 2 weeks, as previously described [12,22].

2.2. Head kidney monocytes/macrophages isolation and bacterial stimulation

Monocytes/macrophages were separated from head kidney of Nile tilapia using the previous descriptions [15,23–26]. Briefly, the head kidneys were removed aseptically and pressed through an 80 μm sterile steel mesh and re-suspended in L-15 medium (Gibico, USA) supplemented with 1% penicillin/streptomycin (Sigma, USA), 10% fetal bovine serum (FBS) (Gibico, USA). The cell suspensions were layered onto a 54%/31% discontinuous percoll (Sigma, USA) density gradient and centrifuged at 400 × *g* at 4 °C for 40 min. The cells were collected and adjusted to a density of 2×10^7 cells/mL, then finally cultured 24 h at 25 °C. After washing away and removing the non-adherent cells, the cells were diluted to 1×10^6 cells/mL with the same culture medium.

The *S. agalactiae* (ZQ0910) [12,27] in the study was from Guangdong Ocean University and the *A. hydrophila* (BYK00810) [22,28] was from the Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, P. R. China. The *S. agalactiae* or *A. hydrophila* was inoculated into brain heart infusion or luriae-bertani broth at 30 °C with shaking at 180 rpm for 6 h. The challenge experiment was performed by formalin-inactivated *S. agalactiae* or *A. hydrophila* in sterile 1 × PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) with a final concentration of 1×10^7 CFU/mL [12,22,27,28]. At 0, 3, 6, 12 and 24 h post-challenges, the cells were collected and immediately lysed by Trizol Reagent (Invitrogen, USA).

2.3. Quantitative real-time PCR

Total RNA and cDNA from the cells was extracted and synthesized using Trizol Reagent and PrimerScript™ RT reagent kit with gDNA Eraser (TaKaRa, Japan) as before descriptions [12,22]. Real-time PCR (qRT-PCR) was performed on the 7500 Real Time PCR System (Life Technologies, USA) using SYBR premix ExTaq™ II (Takara, Japan). The qRT-PCR reaction system and program were the same as previously described [12,22]. The expression of *OnMBL* was normalized by tilapia *β*-actin [12,15,22] using the $2^{-\Delta\Delta Ct}$ method [29], and the data in the monocytes/macrophages were calculated as previously described [12,22]. The primers used are listed in Table 1.

Table 1
Primers used in this study.

Primers	Nucleotide Sequence (5′-3′)	Purpose
β-actin-F	CGAGAGGGAAATCGTGCGTGACA	Control, RT-qPCR
β-actin-R	AGGAAGGAAGGCTGGAAGAGGGC	Control, RT-qPCR
qMBL-F	GGCTCTTTCTCCAAGGCTGTCG	RT-qPCR
qMBL-R	GCGTTGTTCTCCTCCTCGTTCT	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
qIL-10-F	TGGAGGGCTTCCCCGTCAG	RT-qPCR
qIL-10-R	CTGTCGGCAGAACCGTGTCC	RT-qPCR
MIF-F	ACACCAATGTTGCCAGAG	Full cDNA
MIF-R	TCATCATTTAGTCAGTTGTAAGTTC	Full cDNA
qMIF-F	CACATCAACCCTGACCAAAT	RT-qPCR
qMIF-R	GCCTGTTGGCAGCACC	RT-qPCR
qBax-F	GAGCAAGGTGGCTGGGAGG	RT-qPCR
qBax-R	TGCGAATGACAAGAACAGTGGTAAG	RT-qPCR
qBcl-2-F	ACGCAGGCATCCACAGAGTC	RT-qPCR
qBcl-2-R	TCTATCACCTCGGCGAACCTC	RT-qPCR
qFasL-F	TCTCAGCAGAAGATACAGGTCG	RT-qPCR
qFasL-R	TGCCTCTAAGAACAGAATGCGTC	RT-qPCR
qFAIM-F	CGCACTTCATTGTCGGC	RT-qPCR
qFAIM-R	TCGCCATCCAGCAGTAAA	RT-qPCR
qCaspase-3-F	ATCACAGCAACTCAGCCTCTT	RT-qPCR
qCaspase-3-R	GGTTTTCCCACCAGTGATTTA	RT-qPCR

2.4. Inflammation and migration reaction analysis

The experiment was performed according to our previously described method [30] with some modifications. Briefly, the levels of *IL-6*, *IL-10*, *IL-8* and *MIF* were measured in monocytes/macrophages by qRT-PCR. The cells were challenged with (r)OnMBL (5 μg/mL), pET-32a (5 μg/mL), and PBS as control. The (r)OnMBL and pET-32a protein (endotoxin removal) were obtained in our previous study [12].

2.5. Phagocytosis assay

Phagocytosis assays of (r)OnMBL were performed as previous methods [15,30]. Briefly, the monocytes/macrophages phagocytosing *S. agalactiae* and *A. hydrophila* were tested by flow cytometer (BD, USA), which the FITC-labeled bacteria were pre-incubated with TBS (20 mM Tris, 150 mM Nacl, pH 7.4), pET-32a (100 μg/mL) or (r)OnMBL (100 μg/mL). To remove the non-ingested bacteria from the cells, the suspensions were centrifuged over a cushion of 3% BSA in TBS supplemented with 4.5% D-glucose at 100 × *g* at 4 °C for 10 min, repeated three times [15,25,30].

2.6. Respiratory burst assay

To assess the effect of (r)OnMBL on respiratory burst, flow cytometric analysis was performed by referenced to the previous methods [31,32]. The experiment was performed according to the protocol of PMN Oxidative Burst Quantitative Assay Kit (Absin Bioscience, China). The 300 μL monocytes/macrophages suspension (2×10^7 cells/mL) was mixed with 100 μL of (r)OnMBL (final concentration 10 μg/mL), and incubated with a slight shaking at 25 °C for 1 h. Then, 50 μL phorbol ester PMA (100 μg/mL) was added to the 50 μL cell suspension, incubating mixture at 25 °C for 15 min. Subsequently, the mixture was added 25 μL of dihydrorhodamine (DHR, 10 mg/mL), cultured in dark condition at 25 °C for 5 min, then the cells were washed and re-suspended with PBS (repeated three times) before flow cytometer analyses.

2.7. Flow cytometry assay of apoptosis

To explore the effect of (r)OnMBL on apoptosis, flow cytometric analysis was performed by referenced to the previous method [6]. The monocytes/macrophages (1×10^6 cells/mL) were challenged with (r)OnMBL (5 μg/mL and 50 μg/mL), pET-32a (5 μg/mL and 50 μg/mL), and PBS, respectively. All the groups were cultured at 25 °C, and cells were collected at the time of 0, 12, 24, 48 and 72 h post-challenges. Apoptosis experiment was performed by flow cytometry analysis according to the manufacturer's instructions from FITC-Annexin V apoptosis detection kit I (BD, USA). Collected monocytes/macrophages, and then re-suspend cells in 1× binding buffer and adjusted to 5×10^6 cells/mL. The cell suspension (100 μL) was transferred to a 1.5 mL culture tube, and added 5 μL of FITC Annexin V and 5 μL PI, incubating mixture at 25 °C for 15 min in the dark before flow cytometer analyses.

2.8. Assay for effect of (r)OnMBL on apoptosis by quantitative real-time PCR

The monocytes/macrophages were challenged with (r)OnMBL (5 μg/mL and 50 μg/mL), pET-32a (5 μg/mL and 50 μg/mL), and PBS, respectively. The mRNA relative expressions of *Bax*, *Bcl-2*, *FasL*, *FAIM* and *Caspase-3* in the cells were detected and analyzed by qRT-PCR, and the primers are shown on the Table 1.

2.9. Statistical analysis

All data were analyzed using SPSS 17.0 software and represented as mean ± standard deviation of three independent experiments, statistical significance was defined as $p < 0.05$.

3. Results

3.1. The expression patterns of OnMBL after stimulation in vitro

To explore the effects of stimuli on *OnMBL* expression *in vitro*, head kidney monocytes/macrophages were challenged with *S. agalactiae* and *A. hydrophila* (Fig. 1). In monocytes/macrophages, the mRNA levels of *OnMBL* showed significant up-regulation at 6 h post-infection (p.i.) following *S. agalactiae* (7.8-fold) and *A. hydrophila* (8.1-fold) challenges. (Fig. 1).

3.2. Effects of (r)OnMBL on inflammatory and migration reaction

To assay the effects of (r)OnMBL on inflammatory and migration reaction, the mRNA expressions of selected cytokines in monocytes/macrophages were examined. After the (r)OnMBL (5 μg/mL) challenge, the expressions of *IL-6* and *IL-10* were significantly up-regulated. Among, the expression of *IL-6* was raised significantly with the increase of 31.9-fold at 24 h p.i. (Fig. 2A), while the expression of *IL-10* was raised significantly with the increase of 121.7-fold at 12 h p.i. (Fig. 2B). The expressions of *IL-6* and *IL-10* were also up-regulated after pET-32a stimulated (Fig. 2A; 2B); however, the expressions of the *IL-6* and *IL-10* raised much more significantly after the (r)OnMBL challenge compared with the pET-32a stimulation (*IL-6*, 31.9 vs. 1.9-fold, 24 h; *IL-10*, 121.7 vs 1.4-fold, 12 h) (Fig. 2A; 2B).

From the results of *IL-8* and *MIF* mRNA levels after stimulation of (r)OnMBL, the expressions were significantly up-regulated (Fig. 2C; 2D). However, the level of *IL-8* showed significant increase at 24 h p.i, while the *MIF* was significantly increased at 48 h p.i. (Fig. 2C; 2D). The expressions of *IL-8* and *MIF* were also increased after pET-32a stimulated (Fig. 2C; D). However, the expressions of the *IL-8* and *MIF* enhanced more significantly with the up-regulation of 2.2-fold (*IL-8*, 24 h), 3.0-fold (*MIF*, 48 h) after the (r)OnMBL challenge compared with the pET-32a stimulation (Fig. 2C; 2D).

3.3. Promotion of phagocytosis by (r)OnMBL

Monocytes/macrophages were separated to detect the (r)OnMBL induction of phagocytosis by flow cytometer. The results showed that (r)OnMBL could significantly promote monocytes/macrophages phagocytosis. The phagocytic rate of the (r)OnMBL-treated bacteria group was significantly higher than pET-32a or TBS-treated group (Fig. 3B; 3C). These results indicated that (r)OnMBL functioned as an opsonin to enhance monocytes/macrophages phagocytosis.

3.4. Enhancement of respiratory burst activity by (r)OnMBL

To explore the effect of (r)OnMBL on respiratory burst, we treated the monocytes/macrophages using proper concentrations of (r)OnMBL prior to PMA stimulation and determined by flow cytometer. The results showed that the fluorescence cells and the fluorescence intensity of monocytes/macrophages were significantly higher after adding to the (r)OnMBL compared with the control groups (Fig. 4). It showed that the (r)OnMBL could enhance the respiratory burst of monocytes/macrophages.

3.5. Induction of apoptosis by (r)OnMBL

In order to explore whether MBL affects cell apoptosis, we chose FITC-Annexin V/PI double staining method to examine the apoptosis rates of monocytes/macrophages by flow cytometer. The results showed that the level of apoptosis from monocytes/macrophages with

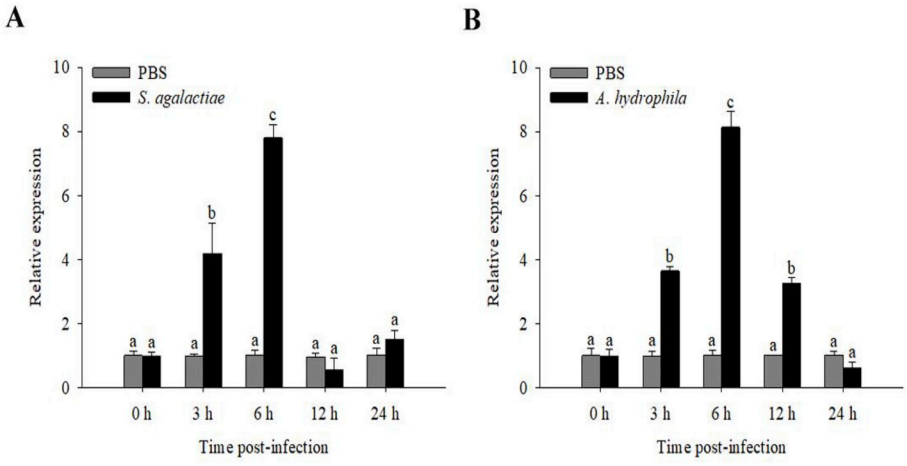

Fig. 1. The mRNA expression of *OnMBL* in head kidney monocytes/macrophages. Nile tilapia head kidney monocytes/macrophages were treated with *S. agalactiae* (1×10^7 CFU/mL), *A. hydrophila* (1×10^7 CFU/mL) and PBS. The mRNA level of *OnMBL* gene was normalized to that β-actin and fold units were calculated deciding the values of the PBS treated cells. The error bars represent standard deviation (n = 3) and different letters (a, b, c) depict statistical significance between groups of tilapia after challenges and health. ($p < 0.05$).

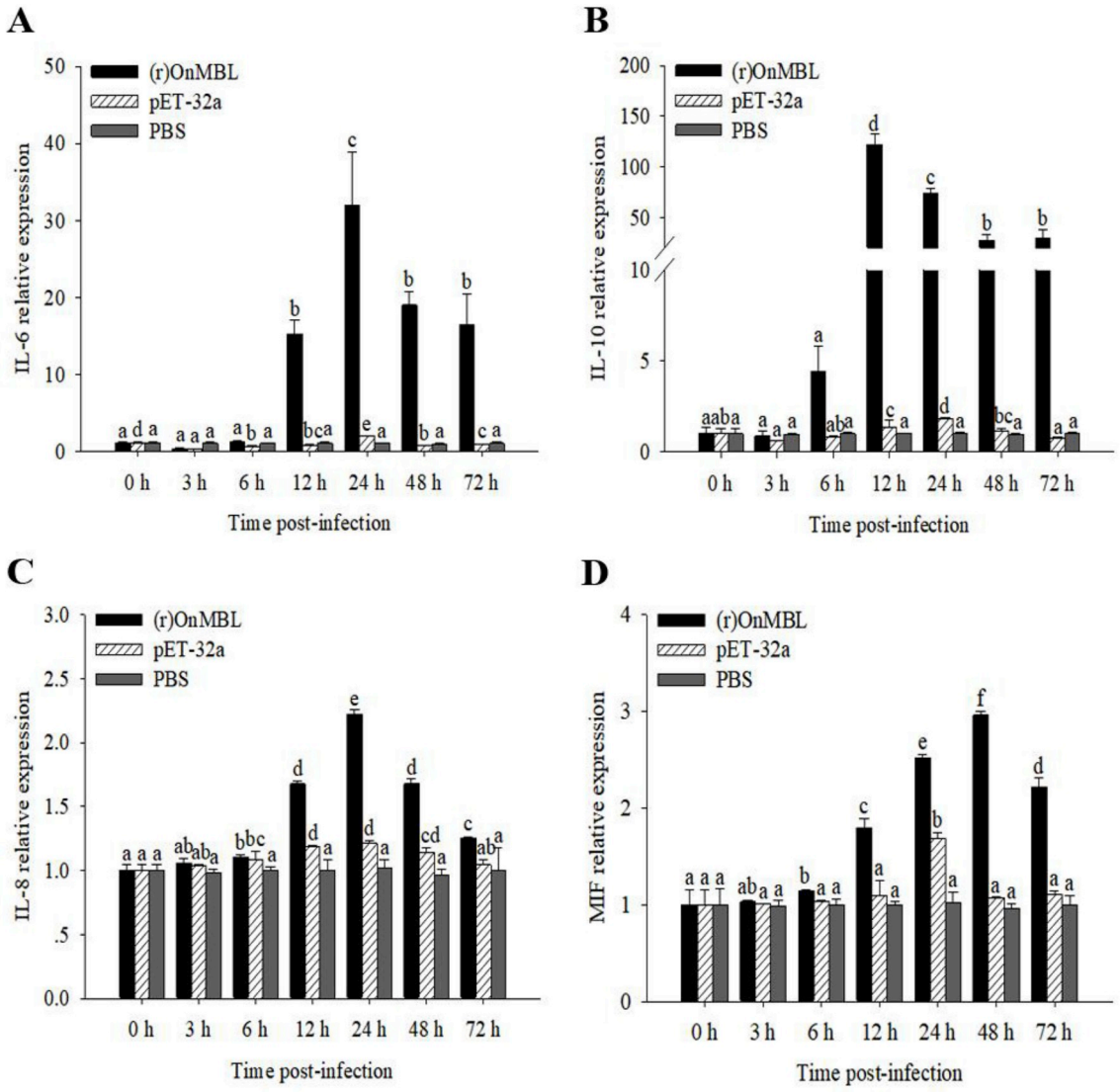

Fig. 2. The mRNA expression of *IL-6* (A), *IL-10* (B), *IL-8* (C) and *MIF* (D) from Nile tilapia in the head kidney monocytes/macrophages. Nile tilapia head kidney monocytes/macrophages were treated with (r)OnMBL (5 μg/mL), pET-32a (5 μg/mL) and PBS. The mRNA level of *IL-6*, *IL-10*, *IL-8* and *MIF* gene was normalized to that of *β*-actin and fold units were calculated deciding the values of the PBS treated cells. The error bars represent standard deviation (n = 3) and different letters (a, b, c) depict statistical significance between groups of tilapia after challenges and health. ($p < 0.05$).

(r)OnMBL at 5 μg/mL or 50 μg/mL was significantly increased in 72 h compared with the control group. The apoptosis rate of cells with (r)OnMBL-treated at 5 μg/mL or 50 μg/mL raised significantly from 0 h to 72 h (Fig. 5A). We found that (r)OnMBL induced apoptosis of monocytes/macrophages with a good dose-and time-dependent relationship (Fig. 5). High concentration of (r)OnMBL could effectively induce apoptosis of monocyte-macrophage cells, and its role was enhanced with the increase of (r)OnMBL concentration and time.

3.6. Regulation of expressions of apoptosis regulators by (r)OnMBL protein

To assay the effect of (r)OnMBL on apoptosis, qRT-PCR was used to analyze the expression changes of *Bax*, *Bcl-2*, *FasL*, *FAIM* and *Caspase*-3 mRNA (Fig. 6). After the (r)OnMBL challenge, the expressions of *Bax, FasL* and *Caspase-3* were significantly up-regulated, and the (r)OnMBL with 50 μg/mL enhanced more prominent with the increase of 63.4-fold (72 h, *Bax*), 2.8-fold (12 h, *FasL*) and 1.9-fold (24 h, *Caspase-3*), respectively (Fig. 6A; 6C; 6E). However, the expression of *FAIM* showed significant down-regulation after the (r)OnMBL challenge, while the *Bcl-2* was significantly down-regulated at 6 p.i. (Fig. 6B; 6D). It showed that (r)OnMBL activated caspase cascade reaction and induced apoptosis by up-regulating *Bax, FasL* and *Caspase-3* gene expression and down-regulating Bcl-2 and *FAIM* gene expression.

4. Discussion

MBL, as a multimeric protein containing collagen-like sequence, plays an important role in innate immunity, to recognize antigen and participate in non-specific cell immunity, such as regulation of inflammation, migration, opsonization, phagocytosis and killing [6,8,30,33]. It also binds to apoptotic cells and may affect apoptosis [6,8]. In this study, we presented the function and characterization of MBL from Nile tilapia. The OnMBL participated in the regulation of inflammation, migration, phagocytosis and enhancement of respiratory burst, and influence on the apoptosis of monocytes/macrophages, which indicated that OnMBL might be involved in host defense against bacterial infection and homeostasis in innate immunity.

Monocytes and macrophages are an essential component of the innate immune system, and possess a multitude of immunological functions, including antigen presentation, phagocytosis and cytokine

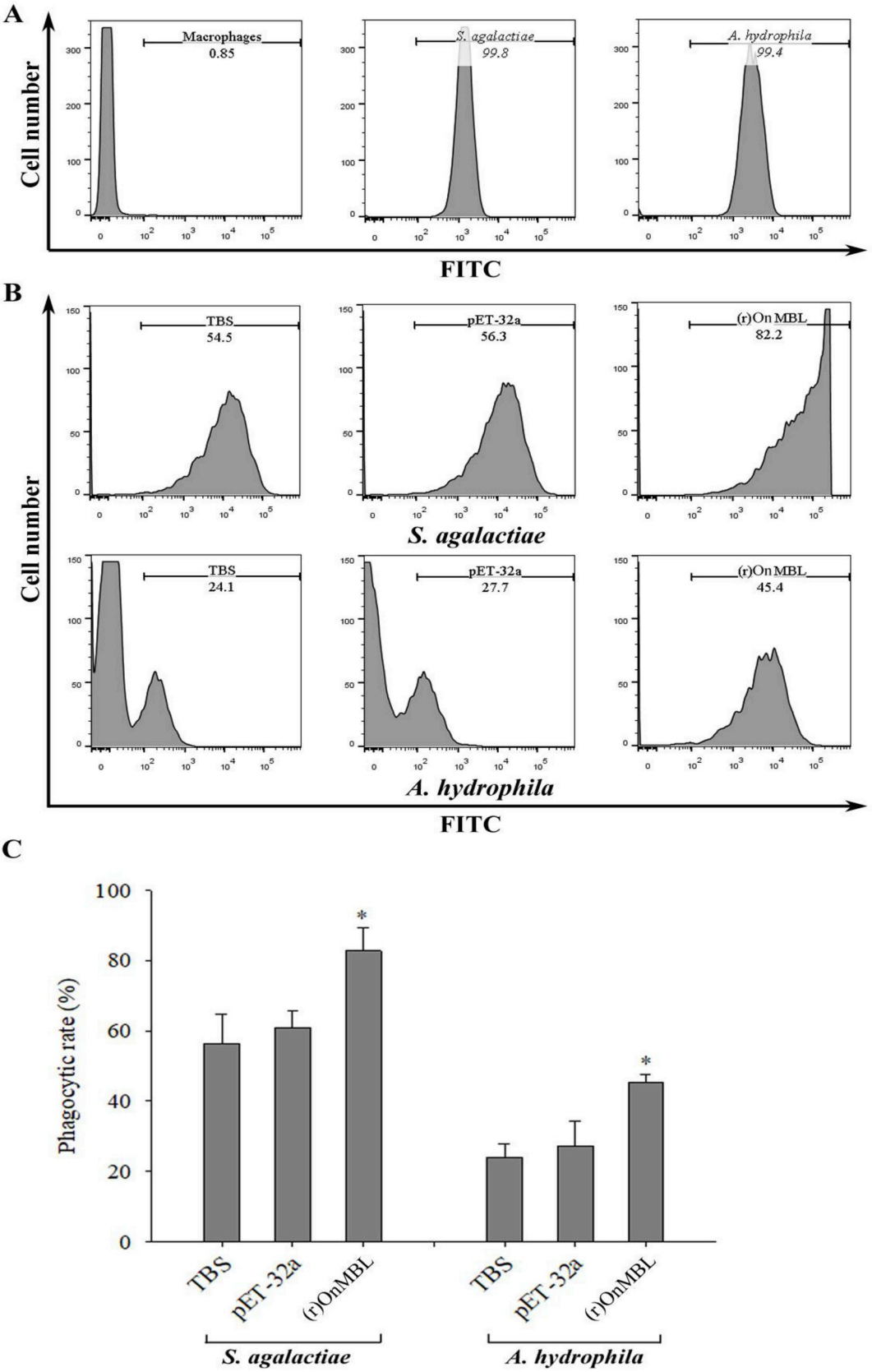

Fig. 3. Effects of (r)OnMBL on phagocytosis of Nile tilapia monocytes/macrophages. Flow cytometric analyses of the monocytes/macrophages phagocytosing *S. agalactiae* and *A. hydrophila* which were treated as described in Section 2. Data show analyses of 10,000 events. (A) The histogram of the cells alone, *S. agalactiae* alone and *A. hydrophila* alone. The marker represented phagocytosis part. (B) The histogram of flow cytometric analyses of the monocytes/macrophages phagocytosing *S. agalactiae* and *A. hydrophila* pre-incubated with TBS, pET-32a protein or (r)OnMBL. The phagocytosis rates were shown near the marker. The results shown here were from one experiment out of three independent experiments. (C) The histogram of the phagocytosis rates. The average standard deviation was obtained from three experiments. The symbol * shows a significant difference from control ($p < 0.05$).

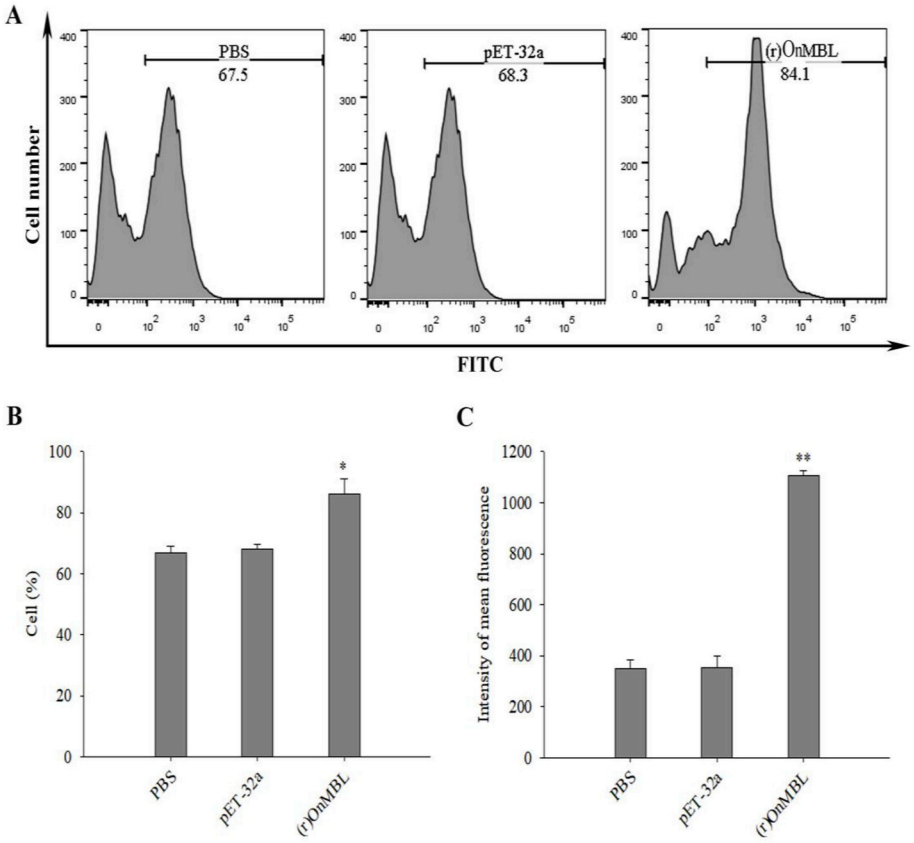

Fig. 4. Flow cytometric analyses of the head kidney monocytes/macrophages respiratory burst from Nile tilapia. (A) The histogram of flow cytometric analyses of the monocytes/macrophages respiratory burst pre-incubated with TBS, pET-32a protein or (r) OnMBL (10 μg/mL). (B) The histogram of the positive cell rates. (C) The histogram of the mean fluorescence intensity. The average standard deviation was obtained from three experiments. Values are shown as mean ± SD (n = 3). $*p < 0.05$, $**p < 0.01$.

production [6]. Additionally, monocytes and macrophages can also characteristically express pattern recognition receptors (PRRs) and some C-type lectins [30,34,35]. In monocytes/macrophages, the expression of *OnMBL* revealed significantly up-regulated post-stimulation. It indicated that the expression of OnMBL was induced by pathogenic bacteria and likely to be involved in host defense against bacterial infection.

The non-specific cellular immune defense, an important component of fish innate defense system, mainly include inflammation, migration, phagocytosis and killing, and is involved in many types of leukocytes, such as monocytes, macrophages and granulocytes [30,36]. Among them, monocytes/macrophages can initiate inflammation and recruit other immune cells, and play an essential role in the non-specific cellular immune defense [6,30,36]. In the immune reaction, the monocytes/macrophages can express and secrete a variety of cytokines, including interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 8 (IL-8, CXCL8), and macrophage migration inhibitory factor (MIF), and participate in the inflammation and migration of the host defense [30,37–40]. Studies have shown that some lectins can participate in inflammatory response [4,30]. In this study, the expressions of the cytokines *IL-6* and *IL-10* were markedly increased after induction of (r) OnMBL with 5 μg/mL concentration. The results were similar to the findings in human MBL and CL-K1 [30,33]. IL-6 is a pleiotropic cytokine with all kinds of biological activity in inflammation, which is rapidly expressed and secreted after stimulation [39,41]. IL-10 is a crucial anti-inflammatory cytokine, which can restrain the release of inflammatory mediators by monocytes/macrophages and enhance the release of anti-inflammatory cytokines to achieve anti-inflammatory effect [40,42]. The results indicated that OnMBL is likely to be involved in regulation of inflammatory reaction.

In inflammation, the migration of phagocytes, including chemokinesis and chemotaxis, enables them to concentrate at the site of infection and plays a crucial biological function and continuing effect, which is crucial for host innate immune defense against pathogens [35,43]. IL-8 is a member of the chemokine family and promotes cell activation, migration and inflammatory regulation [43,44]. MIF is a macrophage migration inhibitory factor, which can restrain the random activity of monocytes/macrophages and play a role in regulating the degree of inflammation by interacting with various cytokines and inflammatory factors in the process of inflammation [37,45]. The expressions of the *IL-8* and *MIF* from monocytes/macrophages were markedly increased after (r)OnMBL stimulation. The results implied that OnMBL is likely to play a crucial role in regulation of the phagocytes migration and inflammation.

Phagocytosis is the process of some specific cells recognition, internalization, killing and digestion of invading microorganisms, which is the basic defense mechanism of organisms [34,36]. Monocytes/macrophages are important immune cells involved in non-specific immune defense and can interact with pathogens related motifs on the surface of exogenous particles through their specific receptors to initiate phagocytosis and clearance [46]. Collectin, an opsonin, is capable of interacting with the surface specific receptors of monocytes/macrophages to promote pathogen phagocytosis [30,47]. In this study, we found that the (r)OnMBL could enhance the phagocytosis of pathogenic bacteria by monocytes/macrophages, which might indicate that OnMBL is likely as an opsonin. It was similar to the previous reports showing that the zebrafish MBL and Nile tilapia CL-K1were able to enhance monocytes/macrophages-mediated phagocytosis [15,30].

Respiratory burst is one of the main ways of phagocytes to perform bactericidal function. When pathogens invade the body, phagocytes are activated and migrated to inflammatory sites, devouring and producing a series of reactive oxygen species (ROS) to kill pathogens [32,48,49]. The respiratory burst of phagocytes is the use of ROS to kill pathogens, and the formation of superoxide ions (O_2^-) and their protonated products, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) [48]. Lectins in teleost fish, such as SmLec1, galectin and CSLs, not only

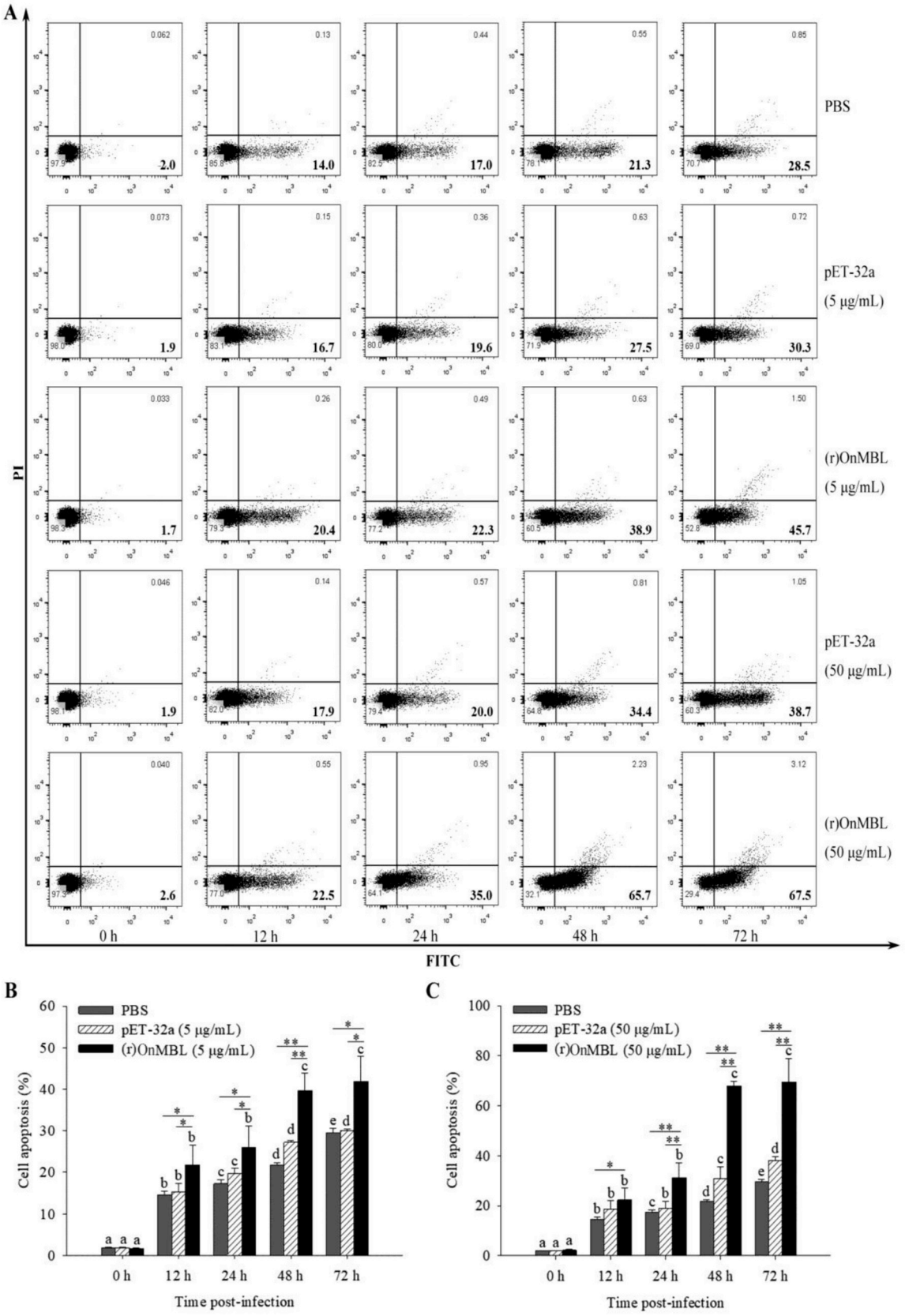

Fig. 5. Effects of (r)OnMBL on apoptosis of Nile tilapia monocytes/macrophages. The induction of apoptosis was determined by Annexin V-FITC/PI double-staining assay. (A) Monocytes/macrophages were treated with (r)OnMBL (5 μg/mL and 50 μg/mL), pET-32a protein (5 μg/mL and 50 μg/mL) and PBS for 0, 12, 24, 48 and 72 h. (B), (C) The histogram of the apoptosis rates. Values are shown as mean ± SD (n = 3) and different letters (a, b, c) depict statistical significance. $*p < 0.05$, $**p < 0.01$.

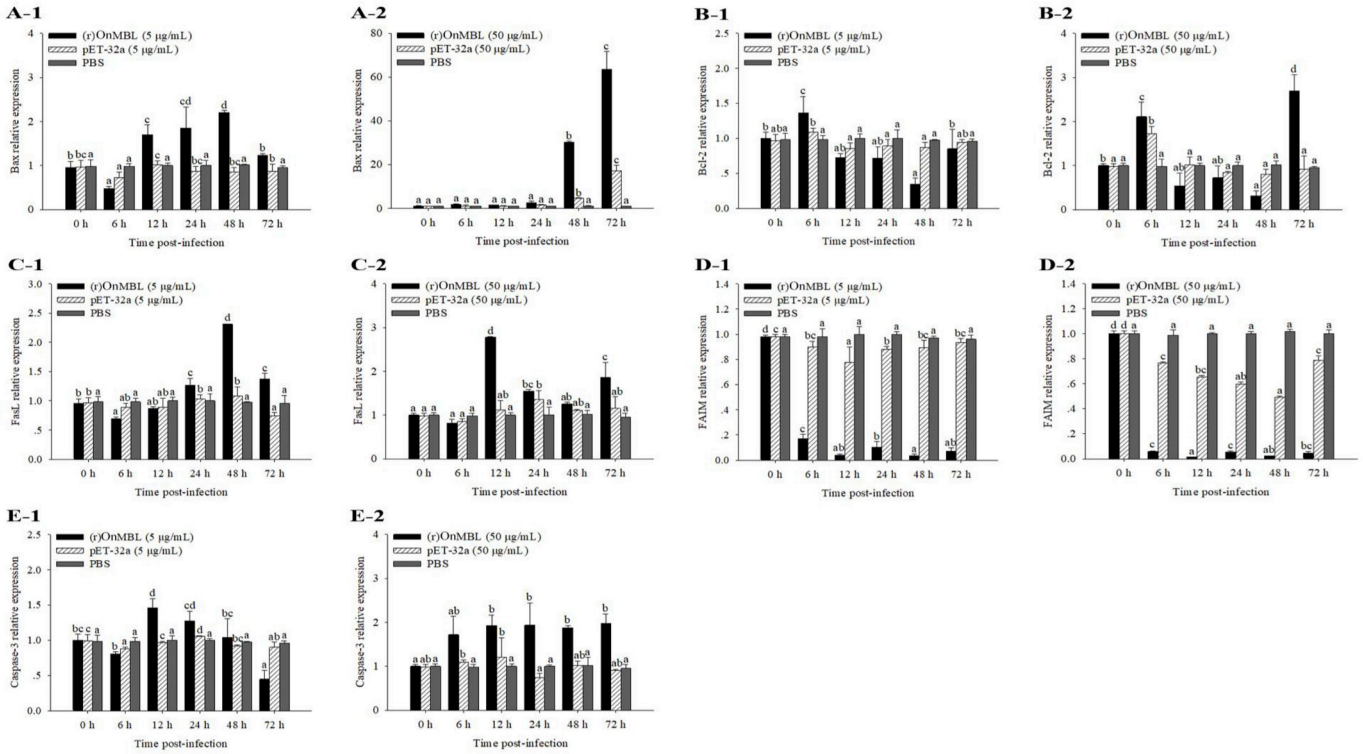

Fig. 6. The mRNA expression of *Bax* (A), *Bcl-2* (B), *FasL* (C), *FAIM* (D) and *Caspase-3* (E) from Nile tilapia in the head kidney monocytes/macrophages. Nile tilapia head kidney monocytes/macrophages were treated with (r)OnMBL (5 μg/mL and 50 μg/mL), pET-32a (5 μg/mL and 50 μg/mL) and PBS. The mRNA level of *Bax*, *Bcl-2*, *FasL*, *FAIM* and *Caspase-3* gene was normalized to that *β*-actin and fold units were calculated deciding the values of the PBS treated cells. The error bars represent standard deviation (n = 3) and different letters (a, b, c) depict statistical significance. ($p < 0.05$).

involve in inflammatory immune regulation, but also enhance the killing of pathogen by macrophages [4]. In this study, we found that OnMBL could increase respiratory burst activity in monocytes/macrophages, indicating that OnMBL may be involved in the killing of pathogens.

One of biological activity in fish lectins is to involve in immune regulation and homeostasis [4]. In order to maintain the stability of internal environment under certain physiological or pathological conditions, the organism regulates the balance between cell proliferation and death, and makes cells actively and orderly death through gene control [6,50]. Apoptosis is a basic biological phenomenon of cells, which refers to the orderly death of cells autonomously controlled by genes in order to maintain homeostasis. In this study, we found that OnMBL could promote apoptosis in monocytes/macrophages, and its effect was enhanced with the increase of concentration and the extension of time at a certain concentration range. It was similar to the previous findings that the human MBL was able to induce apoptosis in the monocytes [6], and C1q, a component like MBL, induced apoptosis of prostate cancer cells [51]. Since the non-infection-induced apoptotic cells were phagocytized by monocytes/macrophages, which could inhibit the activity of monocytes/macrophages, thereby contributing to the regression of inflammation and reducing tissue damage [52].

Apoptosis is not only a special type of cell death, but also has important biological significance and complex molecular biological mechanism. Apoptosis is a process of strict control of multiple genes, which are conserved among species, such as Bcl-2 family, Caspase family and Fas/Fasl gene [53–55]. With the in-depth study of apoptosis mechanism, apoptosis can be roughly divided into two types: caspase-dependent apoptosis and non-caspase-dependent apoptosis. Currently, the two most well understood pathways of apoptosis are death receptor pathway and mitochondrial pathway, which are all through Caspase. Therefore, caspase-dependent apoptosis occupies the main position. Caspase-3, as one of the most important members of caspase family, is in the downstream of cascad of mediation and is an important messenger in the execution of mediation [56,57]. Most of the factors that trigger apoptosis ultimately need caspase-3-mediated signaling pathway to induce cell apoptosis [53,57]. Apoptosis mediated by death receptor pathway is one of the important pathways of cell apoptosis. When apoptosis factors (FasL, TNF-α) bind to apoptosis receptors (Fas, TNFR I, TNFR II), a series of caspases are activated through cascade reactions, leading to cell apoptosis [54,58]. FasL belongs to the TNF family and is a specific ligand of Fas, while FAIM is the Fas apoptosis inhibitory molecule [54,59]. The combination of FasL and Fas mediates cell apoptosis and participates in many physiological and pathological processes in the body [54,58]. In addition, the mitochondrial pathway of apoptosis is mainly regulated by members of the Bcl-2 family. Among them, Bax and Bcl-2 are a pair of important regulatory genes with opposite functions [55]. Their expression proteins are located in mitochondria, which can form dimers to promote or inhibit apoptosis [55,60]. In this study, the expressions of five apoptotic genes (Bax, Bcl-2, FasL, FAIM, and Caspase-3) were examined and analyzed. The data showed that the OnMBL significantly increased the level of pro-apoptotic Bax and FasL, and up-regulated the level of apoptosis executor Caspase-3, while markedly decreased the level of anti-apoptotic Bcl-2 and FAIM in monocytes/macrophages. The results indicated that OnMBL is likely to be involved in regulation of apoptosis response. This findings was similar to that of study on human MBL [6]. According to the above consequence, we speculated that OnMBL may induce apoptosis of monocytes/macrophages by activating death receptor pathway and mitochondrial pathway. Since apoptosis is active programmed cell death, apoptotic cells and apoptotic bodies can be quickly cleared by phagocytes in the body. Thus, the process not only does not stimulate the inflammatory response, but also releases or promotes phagocytes to release a large number of anti-inflammatory factors and activates a series of signal pathways, thereby participating in immune response and maintenance homeostasis. The specific regulation mechanism

remains to be further studied.

In summary, we reported the multi-functions of MBL from Nile tilapia in monocytes/macrophages. The mRNA expressions of *OnMBL* were significantly up-regulated in monocytes/macrophages after *in vitro* bacterial infection (*S. agalactiae* and *A. hydrophila*), indicating that OnMBL might be involved in host defense against bacterial infection. Further, the OnMBL was likely to be involved in regulation of inflammation, migration and enhancement of phagocytosis and respiratory burst activity in monocytes/macrophages. Moreover, the recombinant protein (r)OnMBL could induce the apoptosis of monocytes/macrophages. This study revealed that OnMBL may participate in the regulation of the non-specific cellular immune defense and influence the apoptosis of monocytes/macrophages. Taken together, these findings indicated that OnMBL is likely to be involved in immune regulation, which may play an important role in host defense of innate immunity in Nile tilapia.

Acknowledgements

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

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