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Intelectin mediated phagocytosis and killing activity of macrophages in blunt snout bream (*Megalobrama amblycephala*)

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ABSTRACT

Intelectin, a lectin discovered recently, has been identified in various vertebrate species, such as fish, amphibians, and mammals. In one of our previous studies, the efficient bacteria binding and agglutinating activity of the recombinant *Megalobrama amblycephala* intelectin protein (rMamINTL) and the enhanced immunopositive localization have been observed in the hepatic macrophage-like cells (kupffer cells) post *Aeromonas hydrophila* infection. Thus, the present study primarily focuses on the regulatory effects of rMamINTL on *M. amblycephala* macrophages. This study revealed a prominent LPS-binding activity of rMamINTL and a significantly increased phagocytosis of rMamINTL-treated *A. hydrophila* by *M. amblycephala* macrophages. However, the rMamINTL-treated *M. amblycephala* macrophages exhibited no evident regulatory effect on phagocytosis, whereas the enhanced killing activity of the rMamINTL-treated macrophages was observed, which may be attributed to the induced respiratory burst activity and the expression of inflammatory cytokines. In addition, the anti-proliferation effect of rMamINTL on two tumor cells was observed. However, its mechanism remains to be further studied. In short, these results show that MamINTL is a multifunctional immune protein with effective immunomodulatory activity.

1. Introduction

Host innate immunity derives from the recognition of specific carbohydrates on microbial surface by pattern recognition receptors (PRRs). Lectins are key members of PRRs, which function as phagocytic receptors, soluble opsonins and agglutinins [1,2]. Intelectin is a new-found lectin that was first discovered in *Xenopus laevis* (oocyte lectin XL35) [3,4]. Due to its abundant expression in the mice intestine, the homologous gene of XL35 is termed as "intelectin" (intestinal lectin) [5]. Thereafter, homologs of intelectin have been identified in various animals such as mammals [5,6], fish [7,8], and ascidian [9].

The primary function of intelectins, as agglutinins, is to recognize, bind, and agglutinate the pathogen-specific carbohydrate [6,10–12]. The intelectins identified from amphioxus (*Branchiostoma belcheri tsingtauense*, AmphiITLN239631) and zebrafish (*Danio rerio*, zITLN2) could agglutinate both Gram-positive and Gram-negative bacteria in a Ca^{2+}-dependent manner. In addition, the identified intelectins could comparably bind lipopolysaccharide (LPS) and peptidoglycan (PGN), the major components of Gram-positive and Gram-negative bacterial cell walls, respectively [11,12]. Similarly, the efficient bacteria-binding and agglutinating activity of the recombinant *Megalobrama amblycephala* intelectin protein (rMamINTL) in a Ca^{2+}-dependent manner has been observed in our previous study [8], suggesting the possible roles of macrophages in phagocytosis and killing activity.

In Atlantic salmon, the mannose-binding lectin failed to inhibit *Aeromonas salmonicida* proliferation, while lectin-treated *A. salmonicida* significantly increased the phagocytosis and bactericidal activity of macrophages [13]. Intelectin is a kind of soluble protein secreted into extracellular fluid [6]. Thus, the mouse macrophages phagocytized *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) more efficiently in the medium containing mouse intelectin-1 than in the control medium [10]. In addition, the activation and enhanced killing activity of macrophages could be attributed to the production of ROS (Reactive oxygen species), NO (Nitric oxide), antimicrobial peptides, and lysosomal

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enzymes [14]. However, the mechanism of intelectin-mediated activation and phagocytosis of macrophages is still unknown.

The antiproliferative activity against tumor cells of intelectin has been reported previously [15,16]. For instance, the knockdown of hINTL1 would promote the growth, migration, and invasion of gastric cancer cells *in vitro*, suggesting that hINTL1 played an important role in suppressing tumor progression [15]. Moreover, the major mechanism of the antitumor activity of lectins was reported to rely mainly on promoting the apoptosis of tumor cells via different pathways [17], including NO synthesis by activated macrophages [18,19] and the acceleration of ROS production [20].

M. amblycephala, belonging to *Megalobrama*, Cyprinidae, is one of the major species in the Chinese freshwater polyculture system, which has been under the threat of bacterial septicemia caused by *A. hydrophila* infection in recent years [21]. Our previous study reported that a *MamINTL* gene was cloned and characterized, that this gene was significantly up-regulated upon *A. hydrophila* infection, and that the efficient bacterial binding and agglutinating activity of the recombinant protein was observed [8]. Importantly, the enhanced immunopositive localization was found in the hepatic macrophage-like cells (kupffer cells) post *A. hydrophila* infection. Thus, the possible immune functions of MamINTL in macrophages attracted our attention. Based on these findings, the present study will primarily focus on the regulatory effects of rMamINTL on *M. amblycephala* macrophages, in particular, the effects of rMamINTL on the activation, phagocytosis, and killing activity of macrophages.

2. Materials and methods

2.1. Preparation of the rMamINTL protein

The rMamINTL protein was induced and purified as previously described [8]. Briefly, the encoding region of the *MamINTL* gene was amplified by PCR, and the digested PCR product was inserted into the pre-digested pET32a vector. The recombinant plasmids were transferred into the *Escherichia coli* BL21 (DE3) competent cells (Dingguo, Beijing, China), and the transformants were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) with a final concentration of 0.1 mM at 37 °C for 6 h. Then, competent cells were centrifuged and lysed, and rMamINTL protein was purified using Ni-Agarose His-tagged Protein Purification Kit (CoWin Biosciences, Beijing, China) following the manufacturer's instructions. Endotoxin was removed from the purified rMamINTL using ToxinEraser Endotoxin Removal Kit (GenScript, Nanjing, China), and the removal efficiency was measured using Endotoxin Assay Kit (GenScript) according to the manufacturer's protocol. All procedures were carried out at 4 °C.

2.2. Isolation of head-kidney macrophages

Macrophages were isolated from the head kidney of *M. amblycephala* following the methods described by Ottinger et al. [13]. In brief, the head kidney was removed aseptically, and placed in L-15 medium supplemented with 100 U/mL penicillin and streptomycin, 100 μg/mL gentamicin, 250 μg/mL amphotericin B, and 10 U/mL heparin, and stored on ice for 30 min. Then, the kidney was dissociated using syringe rubber and filtered through a 100-mesh sieve to collect cell suspensions, which were layered upon 34%: 51% Percoll discontinuous gradients and centrifuged at 400 × *g* for 30 min at 4 °C. The macrophages enriched at the 34%: 51% interface were collected and resuspended in 10 mL L-15 medium, then centrifuged at 400 × *g* at 4 °C for 10 min. Re-suspention and centrifugation were repeated one more time. Cells were placed in 96-well (100 μL, 2×10^5 cells per well) or 24-well (200 μL, 1×10^6 cells per well) cell culture plates with L-15 medium containing 10% of fetal bovine serum (Gibco, CA, USA), 100 U/mL penicillin and streptomycin at 28 °C for 4 h. Then, non-adherent cells were washed off and the attached cells were cultured in fresh L-15 medium, and the purity of macrophages was determined by Giemsa staining.

2.3. LPS-binding assay

Each well of the flat-bottomed 96-well microtiter plates (Corning, USA) was added with 5 μg LPS (extracted from *E. coli* 0111:B4, Sigma) dissolved in 100 μL TBS (tris buffered saline) at 4 °C overnight. Nonspecific binding was prevented by additional TBS containing 5% BSA (blocking solution) at 37 °C for 1 h. Various amounts of rMamINTL (2, 8 and 32 ng/μL) in blocking solution were added to the wells at 37 °C for 2 h, respectively. The LPS-bound rMamINTL was detected with rabbit anti-MamINTL antibody (diluted 1: 2000) at 37 °C for 1 h, followed by the incubation with FITC-conjugated goat anti-rabbit IgG (diluted 1: 2000) for 1 h. In the control groups, LPS was replaced by TBS or the anti-MamINTL antibody was replaced by preimmune serum, respectively. The fluorescence intensity of FITC was measured by a fluorescence microplate reader - SpectraMax i3x (Molecular Devices, USA) with excitation/emission spectra at 490/525 nm to calculate LPS-binding activity. The experiments were performed in sextuplicate.

2.4. Bacteriostatic activity of rMamINTL

To analyze the effect of rMamINTL on *A. hydrophila* growth, bacterial density in liquid LB medium was measured at various time points after the treatment of rMamINTL. Bacteria were cultured initially in LB medium at 28 °C until the exponential phase, and diluted (1: 100) using fresh LB medium. Then, rMamINTL was added to the dilution at a final concentration of 32 ng/μL with the heat-inactivated rMamINTL as the control. Samples were incubated at 28 °C and bacterial density was detected by measurement of absorbance at 600 nm at different time points (2 h, 4 h, 6 h, 8 h, and 10 h) post incubation, and the experiment with each sample was conducted in triplicate.

2.5. Effect of rMamINTL on phagocytosis of macrophages

Macrophages incubated in 24-well flat-bottomed microtiter plates (1×10^6 cells per well) were randomly divided into 3 groups: the control group was incubated with PBS (phosphate buffer solution) for 1 h and heat-killed FITC-labeled *A. hydrophila* for 30 min, the rMamINTL-treated macrophage group was incubated with 32 ng/μL rMamINTL for 1 h and heat-killed FITC-labeled *A. hydrophila* for 30 min, and the rMamINTL-treated *A. hydrophila* group was incubated with PBS for 1 h and heat-killed FITC-labeled *A. hydrophila* (pretreated with 32 ng/μL rMamINTL for 1 h) for 30 min, respectively. Then, all groups were washed for 3 times with pre-cooling PBS to remove the non-binding and non-internalized FITC-*A. hydrophila*. Subsequently, trypan blue was used to quench the fluorescence from the remaining non-internalized FITC-*A. hydrophila* [22]. Thereafter, the phagocytic activity of macrophages was measured.

For quantitative analysis, samples were stained with DAPI and washed with PBS for 3 times, and lysed in cell lysis buffer (Beyotime, China). Then, the fluorescence intensity of FITC and DAPI was also measured by the fluorescence microplate reader - SpectraMax i3x. This fluorescence intensity with the excitation/emission spectra of DAPI at 358/460 nm represented the amount of macrophages. The phagocytosis of macrophages was presented as mean fluorescence intensity (FITC/DAPI) in comparison with the control. For visual analysis, the samples were fixed with 4% paraformaldehyde for 10 min, stained with DAPI, and washed for 3 times with PBS, then imaged using a fluorescence microscope.

2.6. Effect of rMamINTL on killing activity of macrophages

Macrophages were incubated in 96-well flat-bottomed microtiter plates (2×10^5 cells per well) for adhesion and differentiation. Then,

macrophages incubated with or without rMamINTL (32 ng/μL, containing 5 mM Ca^{2+}) were challenged with *A. hydrophila* at a multiplicity of infection (MOI) of 10 for 30 min, and washed with prewarmed medium containing 0.5 mg/mL gentamicin for 3 times. Then, macrophages were cultured in the medium containing 0.5 mg/mL gentamicin for 90 min and lysed with 1% Triton-X 100. The surviving bacteria were washed with PBS for 3 times, and then measured with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay. This assay was performed in triplicate and the results were expressed as survival percentage.

2.7. Measurement of reactive oxygen species (ROS) production

Reactive oxygen species (ROS) production of *M. amblycephala* macrophages was detected using the Nitro Blue Tetrazolium (NBT) reduction test [23]. Macrophages in 96-well plate (2×10^5 cells per well) were incubated with 100 μL PBS containing 0.1% of NBT (Sigma, St. Louis, USA) and 5 μg/mL rMamINTL at 24 °C for 12 h. The reaction was terminated with 80 μL of 70% methanol. The *M. amblycephala* macrophages were air-dried, and the produced formazan was subsequently dissolved in 90 μL of 2 M KOH and 110 μL of DMSO. Optical density was measured by a spectrophotometer at 620 nm with KOH/DMSO as the blank.

2.8. RNA isolation

Total RNA was extracted using Trizon reagent (CoWin Biosciences, Beijing, China) according to the manufacturer's instructions. The quality and concentration of total RNA were measured by agarose gel and NanoDrop 2000 (Thermo Scientific, Delaware, USA), respectively. The first strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's protocol and stored at −20 °C for further use.

2.9. Quantitative real-time PCR analysis

The expression patterns of the target genes were analyzed using quantitative real-time PCR (qRT-PCR) as previously described [24]. Briefly, qRT-PCR was performed in a LightCycler® 480 II real-time PCR detection system (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics) according to the manufacturer's protocol. Relative expression levels of the target genes were measured in terms of threshold cycle value (Ct) by the $2^{-\Delta\Delta Ct}$ method [25] using *18S rRNA* as the internal reference [26–28]. All reactions were performed in triplicate. The primers were listed in Table 1, and the standard curves were presented in the Supplemental Fig. 1. The expression levels of the control were set as 1, and the relative expression levels were indicated as fold change.

2.10. EPC and HeLa cell proliferation assay

The effect of rMamINTL on proliferative activity of L8824 (Grass Carp Hepatic Cell), EPC (Epithelioma Papulosum Cyprini) and HeLa cells was determined as described by Wong and Ng [29]. Briefly, EPC and HeLa cells were suspended in RPMI medium with the cell density adjusted to 2×10^4 cells/mL. And 100 μL of cell suspension was added into each well of 96-well plate, followed by incubation for 24 h. The 100 μL of rMamINTL dissolved in RPMI medium at final concentrations of 2, 8 and 32 ng/μL was then added to the wells. After incubation for 24 h, 20 μL of 5 mg/mL MTT formazan dissolved in PBS was added into each well and incubated for 4 h. The plates were then shaken at 2500 rpm for 5 min. The supernatant was carefully removed, and 150 μL of dimethyl sulfoxide was added to each well to dissolve the MTT formazan. After 10 min, the absorbance at 570 nm was measured by a microplate reader - SpectraMax i3x.

Table 1
Primers used for qRT-PCR in the present study.

Primer Name	Primer Sequence (5′-3′)	Amplification efficiency
qTNFα-F1	CGCTGCTGTCTGCTTCAC	91.4%
qTNFα-R1	CCTGGTCCTGGTTCACTC	
qiNOS-F1	GTATCATGACAGCTTACTCTAGG	92.9%
qiNOS-R1	GACTAAGATTTCCTGGATAGTGG	
q18S rRNA-F1	CGGAGGTTCGAAGACGATCA	99.4%
q18S rRNA-R1	GGGTCGGCATCGTTTACG	

2.11. Statistical analysis

In the present study, all data were presented as mean ± SE. The statistical significance was assessed by one-way analysis of variance (ANOVA) using SPSS 17.0, with $P < 0.05$ considered as significant difference and $P < 0.01$ as extremely significant difference.

3. Results

3.1. LPS-binding activity of rMamINTL

To explore the Ca^{2+}-dependent bacteria-binding activity of rMamINTL, LPS-binding assay was performed to quantify the recognition and binding of rMamINTL to LPS, known as the lipoglycans and endotoxins in the outer membrane of Gram-negative bacteria. As shown in Fig. 1, the LPS-binding curves of rMamINTL showed a dose-dependent tendency, and an efficient LPS-binding activity was observed when the concentration of rMamINTL was higher than 2 ng/μL.

3.2. Bacteriostatic activity of rMamINTL

The inhibitory effect of rMamINTL on bacterial growth was detected to determine the direct bacteriostatic activity. As shown in Supplemental Fig. 2, the addition of rMamINTL showed no significant effect on bacterial abundance (absorbance at 600 nm), indicating that rMamINTL had no significant inhibition effect on bacterial growth. Therefore, it could be inferred that rMamINTL possessed no efficient

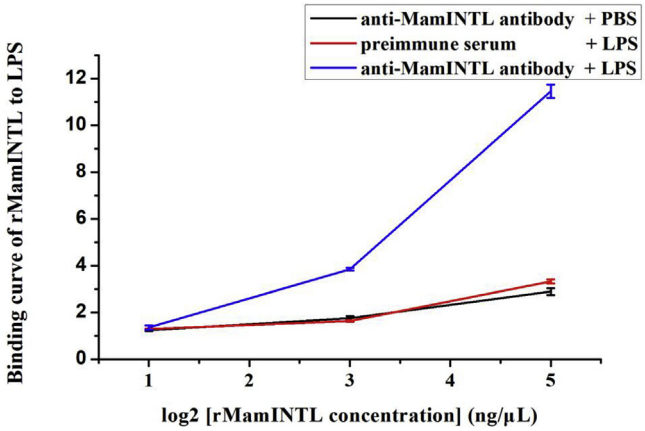

Fig. 1. Quantitative binding curves of rMamINTL to LPS. The microtiter plates were coated with 5 μg of LPS and then incubated with rMamINTL at different concentrations in the presence of Ca^{2+}. Then, specific anti-MamINTL antibody and FITC labeled secondary antibody was added sequentially to determine the binding activity between rMamINTL and LPS. In addition, LPS was replaced by PBS or anti-MamINTL antibody was replaced by preimmune serum in two control groups, respectively. The fluorescence intensity of FITC (absorbance at 490 nm) was measured by a microplate reader - SpectraMax i3x and the data were presented as mean ± SE (n = 3).

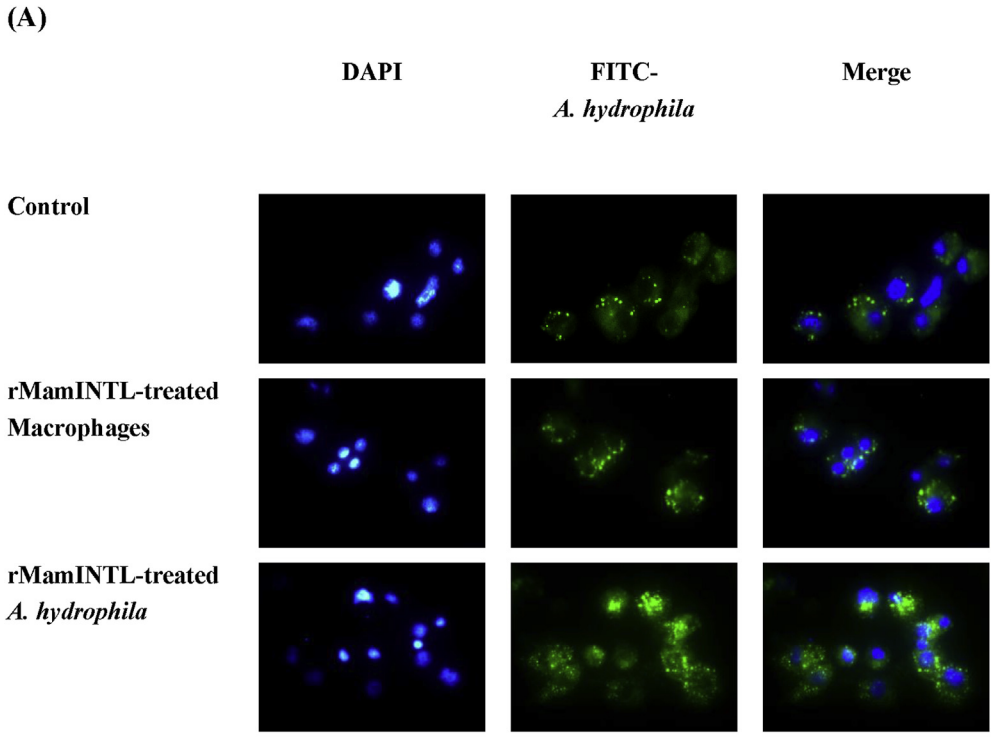

Fig. 2. Effect of rMamINTL on the phagocytosis of *M. amblycephala* macrophages. Macrophages in the 24-well flat-bottomed microtiter plates were randomly divided into 3 groups: the control group, the rMamINTL-treated macrophage group, and the rMamINTL-treated *A. hydrophila* group. (**A**) For visual analysis, cells were fixed with 4% paraformaldehyde for 10 min and stained with DAPI, then imaged using a fluorescence microscope. The 3 columns of images represented stained macrophage nuclei with DAPI, phagocytized FITC-*A. hydrophila*, and merged images, respectively. (**B**) For quantitative analysis, samples were stained with DAPI and measured by the fluorescence microplate reader. The phagocytosis of macrophages was presented as mean fluorescence intensity (FITC/DAPI) in comparison with the control. The fluorescence intensity of FITC (absorbance at 490 nm) and DAPI (absorbance at 358 nm) was measured by a microplate reader - SpectraMax i3x and the values were described as mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

bacteriostatic activity.

3.3. Effect of rMamINTL on phagocytosis of macrophages

Many isolated cells were found to be adhered to the cell culture plate within 4 h. Giemsa staining indicated that more than 95% of the adherent cells were macrophages which were irregular in shape with cell diameters of 10–20 μm and nuclei diameters of 5–7 μm (Supplemental Fig. 3). As shown in Fig. 2, the phagocytic fluorescence intensity of the rMamINTL-treated *A. hydrophila* (heat-killed and FITC-labeled) was significantly higher than that of the control, indicating that treatment of *A. hydrophila* with rMamINTL could induce the phagocytosis of *M. amblycephala* macrophages. On contrast, no significant difference in the phagocytosis was observed between the rMamINTL-treated macrophage group and the control group.

3.4. rMamINTL-treated killing activity of macrophages

The *M. amblycephala* macrophages were activated after incubation with rMamINTL (data not shown), while no significant difference in phagocytosis was observed. Thus, the effect of rMamINTL on the killing activity of macrophages was assayed. The results showed (Fig. 3) that survival percentage of the internalized *A. hydrophila* in the rMamINTL-treated macrophages group was significantly decreased, indicating the increased killing activity of macrophages post rMamINTL treatment.

3.5. rMamINTL-induced the expression of cytokines

The expressions of two cytokines, *TNFα* (tumor necrosis factor alpha) and *iNOS* (inducible nitric oxide synthase), were detected in the present study to analyze the induction effect of rMamINTL on *M. amblycephala* macrophages. As shown in Fig. 4, the expressions of *TNFα*

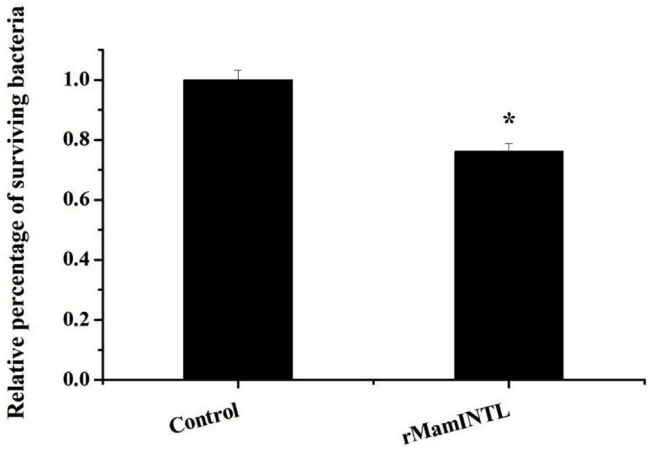

Fig. 3. The effect of rMamINTL on the killing activity of macrophages. The result was presented as the survival percentage of *A. hydrophila* detected using MTT method, and the absorbance at 570 nm was measured by a microplate reader - SpectraMax i3x. The data were presented as mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

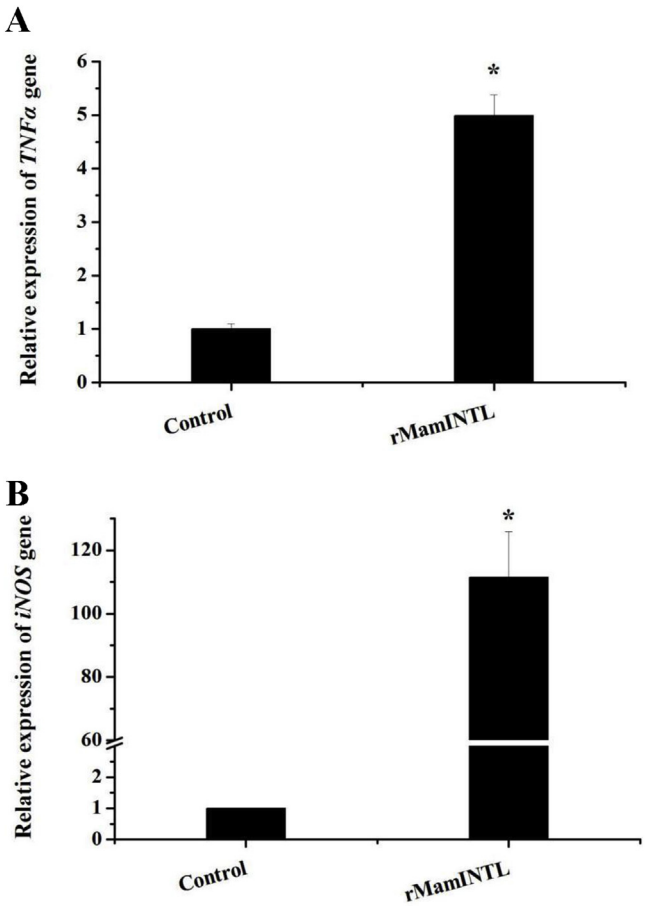

Fig. 4. Expression patterns of the *M. amblycephala TNFα* (A) and *iNOS* (B) genes post incubation of macrophages with rMamINTL protein, and *18S rRNA* is served as an internal control. Data were shown as mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

and *iNOS* genes were significantly up-regulated post incubation with rMamINTL, indicating the activated regulatory effect of rMamINTL on macrophages.

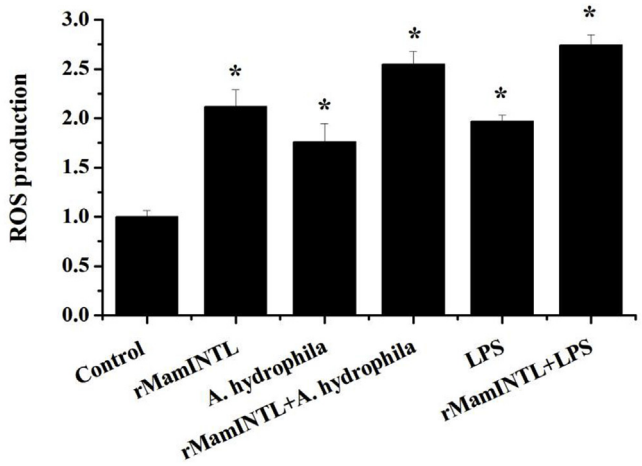

Fig. 5. Reactive oxygen species (ROS) production of the *M. amblycephala* macrophages was detected using NBT reduction assay. The absorbance at 620 nm was measured and described as mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

3.6. rMamINTL-induced respiratory burst of macrophages

The killing activity of macrophages was always associated with respiratory burst level, and the ROS production was determined using NBT reduction assay in the present study. As shown in Fig. 5, the ROS level of the rMamINTL-treated group was significantly increased, compared to that of the control. Similarly, the enhanced ROS levels were also observed in *A. hydrophila*-challenged group and LPS-treated group, as well as in the presence of both rMamINTL and *A. hydrophila* (or LPS).

3.7. Effect of rMamINTL on proliferation of EPC and HeLa cells

As shown in Fig. 6, the proliferation of two tumor cell lines (EPC and HeLa) were inhibited post incubation with rMamINTL, while no significant effect on the proliferation of the normal cell line (L8824) was observed. In addition, the regulation effect was concentration-related, that is, the inhibitory effect was more significant at high concentration levels. Specifically, a 20% decrease of HeLa cell proliferation rate was obtained at the concentration of 32 ng/μL, while a 30%

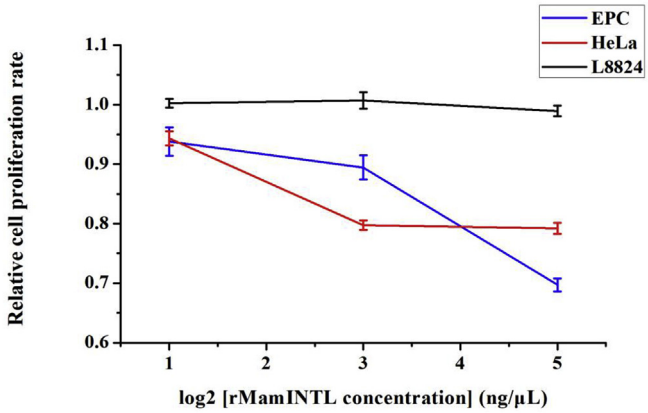

Fig. 6. Inhibitory effect of rMamINTL protein on the proliferation of two tumor cell lines, EPC (Epithelioma Papulosum Cyprini) and HeLa. The proliferative activity was determined using MTT assay, and the absorbance at 570 nm was measured by a microplate reader - SpectraMax i3x. A normal cell line L8824 (Grass Carp Hepatic Cell) was served as the control, and the data were presented as mean ± SE (n = 3).

decrease of EPC cell proliferation rate was observed at the same concentration.

4. Discussion

Intelectin is a newly discovered Ca^{2+}-dependent lectin, which was first reported in *X. laevis*, termed as XL35 [3], and the characterization and expression analysis have been reported in several species including cephalochordates, fish, amphibians, and mammals. In a previous study, we cloned and characterized a *MamINTL* gene, examined its expression and localization post *A. hydrophila* infection, and investigated the bacteria binding and agglutinating activity of the recombinant MamINTL protein [8]. The LPS-binding assay was performed to assess the interaction between rMamINTL and bacterial cell wall components. The effective LPS-binding activity of rMamINTL was observed in this study, which might contribute to explaining the efficient bacteria binding of rMamINTL reported in our previous study [8]. Ca^{2+} is essential for bacterial agglutination of rMamINTL, but not for bacteria and LPS binding. This study found that the presence of Ca^{2+} increased the recognition and binding ability of rMamINTL on bacteria and LPS. This finding was consistent with that of a previous study of amphioxus [30]. Based on these findings, it could be speculated that although Ca^{2+} is not essential for carbohydrate recognition, it is essential for the formation of interaction complexes between bacteria and intelectin, which can promote the agglutination of bacteria through intelectin.

Our finding that incubation of *A. hydrophila* with rMamINTL had no significant inhibitory effect on bacterial proliferation, indicating no bacteriostatic activity of rMamINTL, which was in line with the findings of a previous study of mannose-binding lectin in Atlantic salmon [13]. However, a strong immunopositive localization in the hepatic macrophage-like cells (kupffer cells) post *A. hydrophila* infection has been reported in our previous study [8]. Based on it, the present study primarily focused on the regulatory effects of rMamINTL on the *M. amblycephala* macrophages. The phagocytosis of rMamINTL-treated *A. hydrophila* by *M. amblycephala* macrophages was observed to be significantly higher than that of the control, which agreed with the report on the lectin-incubated *A. salmonicida* in Atlantic salmon [13]. Therefore, rMamINTL could be assumed to first bind and agglutinate bacteria, then to form the interaction complexes, and then to further interact with the receptors of rMamINTL on the surface of macrophages, finally to facilitate the phagocytosis of macrophages.

However, the rMamINTL-treated *M. amblycephala* macrophages had no significant effect on the phagocytosis in this study. Our results were different from the results of previous studies of the mouse intelectin-1 [10] and *M. amblycephala* transmembrane lectin (mannose receptor serving as PRR) [31]. The different regulatory effects on the phagocytosis of bacteria between the *M. amblycephala* and mouse intelectins may be attributed to their different functions and interactions with macrophages in the evolution of diverse species [32]. Whereas, activated macrophages and enhanced killing activity were observed post incubation with rMamINTL in the present study. The production of ROS, NO, antimicrobial peptides, and lysosomal enzymes was important for the killing activity of macrophages [14]. Thus, the enhanced respiratory burst activity and expression of the *iNOS* and *TNFα* genes post treatment of rMamINTL should contribute to the enhancement of bactericidal effect of macrophages. It was reported that both increased expression of the *iNOS* and *TNFα* mRNA and induced respiratory burst activity were typical characterization of M1 polarization of fish macrophages [33]. Thus, it could be speculated that incubation of the *M. amblycephala* macrophages with rMamINTL could trigger the M1 polarization of macrophages.

The anti-proliferative activity of intelectins against tumor cells have been reported previously [15,16]. Similarly, the anti-proliferation effect of rMamINTL on two tumor cells (HeLa and EPC) was observed in the present study. It was reported previously that the main mechanism of the antitumor activity of lectins depended on their ability to induce apoptosis via multiple pathways [17]. However, the mechanism of the fish intelectins was still unclear. Thus, further studies should be conducted in more tumor cell lines to verify the anti-proliferation effect of rMamINTL so as to reveal its anti-tumor mechanism.

In summary, the efficient LPS-binding activity of rMamINTL was observed in the present study, which contributes to explaining its bacteria-binding activity detected previously. The phagocytosis of rMamINTL-treated *A. hydrophila* by *M. amblycephala* macrophages was found to be significantly increased, while the rMamINTL-treated macrophages showed no significant effect on the phagocytosis of *A. hydrophila*. Nevertheless, treatment with rMamINTL could enhance the killing activity of macrophages, which may be attributed to the enhanced respiratory burst activity and expression of the *iNOS* and *TNFα* genes. In addition, the anti-proliferation effect of rMamINTL on two tumor cells was observed, while its mechanism remains to be further studied. In a word, MamINTL is a multifunctional immune protein with effective immunomodulatory activity.

Acknowledgments

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Appendix A. Supplementary data

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