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MicroRNA-155 promotes pro-inflammatory functions and augments apoptosis of monocytes/macrophages during *Vibrio anguillarum* infection in ayu, *Plecoglossus altivelis*

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ABSTRACT

Upon recognition of pathogen-associated molecular patterns by pattern-recognition receptors, immune cells are recruited, and multiple antibacterial/viral signaling pathways are activated, leading to the production of immune-related cytokines, chemokines, and interferons along with further activation of the adaptive immune response. MicroRNAs (miRs) play essential roles in regulating such immune signaling pathways, as well as the biological activities of immune cells; however, knowledge regarding the roles of miRs in the immune-related function of monocytes/macrophages (MO/MΦ) remains limited in teleosts. In the present study, we addressed the effects of miR-155 on *Vibrio anguillarum*-infected MO/MΦ. Our results showed that miR-155 augmented MO/MΦ expression of proinflammatory cytokines and attenuated the expression of anti-inflammatory cytokines. Additionally, the phagocytosis and bacteria-killing abilities of these cells were boosted by miR-155 administration, which also promoted M1-type polarization but inhibited M2-type polarization. Furthermore, the *V. anguillarum*-infection-induced apoptosis was also enhanced by miR-155 mimic transfection, which might have been due to excessive inflammation or the accumulation of reactive oxygen species. These results represent the first report providing a detailed account of the regulatory roles of miR-155 on MO/MΦ functions in teleosts and offer insight into the evolutionary history of miR-155-mediated regulation of host immune responses.

1. Introduction

The innate immune system constitutes the first line of defense against microbial pathogens and is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns by pattern-recognition receptors (PRRs). Among these, Toll-like receptors (TLRs) comprise one of the most important classes associated with bacteria clearance and exhibit the most extensive spectrum of PAMP recognition. For example, TLR1 is involved in the recognition of triacylated lipoproteins and mycobacterial products in concert with TLR2 to form a heterodimer [1], mammalian TLR4 is responsible for lipopolysaccharide (LPS) recognition [2], TLR5 signaling is triggered by binding with the bacterial flagellum [3], and TLR9 recognizes unmethylated CpGs in bacterial DNA and initiated antibacterial signaling pathways [4]. TLR-signaling pathways are classified into two distinct types: myeloid-differentiation primary response protein 88 (MyD88)-dependent pathways and Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing interferon (TRIF)-dependent pathways [5]. The MyD88-dependent response is utilized by almost all TLRs, with the exception of TLR3. Upon ligand recognition and TLR dimerization, MyD88 binds to the TIR domain of the corresponding TLR, after which IL-1 receptor-associated kinase 4 (IRAK4) is recruited to MyD88, and tumor necrosis factor (TNF) receptor-associated factor 6 along with the TAK1/TGF-β-activated kinase (TAB) complex are activated in sequence [6]. This is followed by activation and nuclear translocation of the transcription factor nuclear factor kappaB (NF-κB), which induces the transcription of genes encoding inflammatory cytokines [7]. The TRIF-dependent pathway is considered specific for only a few TLRs, such as TLR3 and TLR4 in mammals, and TRIF can activate either TANK-binding kinase 1 (TBK1) or receptor-interacting serine/threonine kinase 1 (RIPK1), which creates a branch in the signaling pathway [8,9]. The TRIF/TBK1 signaling complex phosphorylates interferon-regulatory factor (IRF)-3, allowing its translocation to the nucleus and the concomitant

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production of type I interferon. Activation of RIPK1 causes a series of signal-transduction events in the same manner as that of the MyD88-dependent pathway [10].

MicroRNAs (miRs) are noncoding RNAs with the length of ~23 nt that are highly conserved during evolution. Mature miRs bind to the 3′ untranslated region (UTR) of target cellular mRNAs to degrade mRNA or inhibit translation [11]. Numerous miRs reportedly play crucial roles in macrophage biological processes, with the inflammatory responses of macrophages being regulated by a distinct set of miRs. miR-21, -27a, −92a, −124, −147, −210, −125a-5p, and −4661 can dampen TLR-induced signaling and cytokine production to prevent excessive pro-inflammatory responses [12–21], whereas miR-17/20a/106a and miR-98 positively regulate the TLR-initiated immune response of macrophages, and miR-155 exhibits both pro- and anti-inflammatory effects by regulating suppressor of cytokine signaling 1 (SOCS1) and MyD88, respectively [12,22–25]. Additionally, macrophage phagocytosis and polarization are also regulated by a cluster of miRs, with miR-33 along with miR-124, -127, −155, −511, and/or let-7c modulating macrophage polarization by activating or inhibiting relative signaling pathways, and miR-125a-5p, −27a/b, and −17/20a/106a regulating processes associated with phagocytosis [12,20,26–30]. Moreover, miR-125a-5p regulates lipid uptake, as well as oxysterol-binding-protein-related protein 9 expression, in oxidized low-density lipoprotein-stimulated monocytes/macrophages (MO/MΦ), miR-27a/b regulates cellular cholesterol efflux, influx, and esterification/hydrolysis, miR-155 is involved in lipid uptake in THP-1 macrophages, and miR-33 regulates macrophage autophagy and promotes the progression of atherosclerosis. These findings indicate that macrophage-derived miRs might also affect process associated with lipid metabolism [27,29,31,32].

Despite in-depth studies of the regulatory roles of miRs in mammals, detailed knowledge regarding their function in teleost macrophages is limited, although some studies have focused on the roles of teleost miRs during infection and inflammation. As important PRRs for viral RNA, retinoic acid-inducible gene-1-like receptors (RLRs) play essential roles in antiviral immune responses. To date, several miRs reportedly interact with essential components of this antiviral signaling pathway to regulate immune responses in teleosts. For example, miR-3570 targets *mitochondrial antiviral-signaling protein* (*MAVS*), miR-210 targets *OTU deubiquitinase 5* and *transmembrane protein 173* (encoding STING), and miR-145 targets *melanoma-differentiation-associated protein 5* to negatively regulate RLR signaling [33–36]. Alternatively, miR-122 and miR-214 target *dihydroxyacetone kinase* and *AMP-activated protein kinase*, respectively, to promote antiviral responses [37,38]. Additionally, miR-132-3p, −155, −181a-3p, −184, −19a-3p, −200b-3p, −30a-5p, −454b, -727-3p, and -737-3p are potentially involved in regulating RLR signaling, although the exact mechanisms and targets require further characterization [39].

Regarding the regulation of TLR-signaling pathways and NF-κB activation in teleosts, miR-115 and miR-142a-3p target *TLR5* while miR-21 targets *TLR28* to negatively regulate TLR signaling by directly degrading these pathogen-specific receptors [40,41]. MiR-214, -19a, −3570, and −148 target *MyD88*, and miR-203 targets *IRAK4* to inhibit the expression of essential TLR-signaling adaptors [42–46]. Furthermore, miR-375 targets the regulatory factor *dual-specificity phosphatase 1*, and miR-216a targets the transcription factor *p65* to dampen TLR-initiated inflammatory responses [47,48]. In addition to immune-related processes, miRs in teleosts are associated with embryogenesis, growth, and muscle quality, suggesting their substantial roles in teleost-specific cellular and biological processes [49–51].

Ayu (*Plecoglossus altivelis*) is an economically important fish that is widely cultured in East Asia; however, the development of ayu aquaculture has been challenged by bacterial and viral fish diseases that have caused problems associated with production and animal welfare [52]. Given the importance of macrophages during infection and inflammatory responses, and because miRs constitute essential regulators of macrophage function, characterization of miR-related regulatory mechanisms in macrophages is necessary. Previous transcriptome data associated with ayu head kidney-derived MO/MΦ indicated that miR-155 exhibited the highest upregulation among miRs following *Vibrio anguillarum* infection, with the sequence of miR-155 being the same as that of its mammalian counterpart. In the present study, the roles of miR-155 in macrophage-specific inflammatory responses and apoptosis during *V. anguillarum* infection were characterized in detail in ayu. We determined the effects of *miR-155* overexpression on *IL-1β*, *TNF-α*, *IL-10*, and *TGF-β* levels during *V. anguillarum* challenge, Additionally, we evaluated the effects of miR-155 transfection on the promotion of phagocytosis, bacterial killing, and the polarization of ayu MO/MΦ, as well as effects on *V. anguillarum*-induced apoptosis. To the best of our knowledge, this represents the first report elucidating the positive regulatory roles of miR-155 on the proinflammatory-related functions of teleost MO/MΦ.

2. Materials and methods

2.1. Samples and challenge

Healthy ayu (45.0 ± 2.4 g) were purchased from a fishery in Ninghai County (Ningbo, China) and temporarily kept in a recirculating water system (21.0 ± 1.0 °C) for 2 weeks, as previously described [53]. *V. anguillarum* challenge was performed, as previously reported [54]. Briefly, 36 healthy ayu were randomly divided into two groups. The fish in the experimental group were intraperitoneally injected with 1.2×10^4 colony forming units (CFUs) of live *V. anguillarum* in 100 μL PBS, whereas fish in the control group were injected with PBS alone. Ayu were sacrificed at 0-, 4-, 8-, 12-, 24-, or 48-h post-infection (hpi), and the gill, head kidney, intestine, liver, and spleen were collected and stored at −80 °C for later use. Healthy ayu tissues, including the heart, brain, muscle, intestine, skin, head kidney, liver, gill, and spleen, were also collected for further tissue expression profile analysis. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

2.2. RNA extraction and real-time quantitativePCR (RT-qPCR)

Total RNA of both ayu tissues and MO/MΦ were isolated using RNAiso (TaKaRa, Dalian, China), treated with DNase I (TaKaRa), and reversed transcribed into first-strand cDNA using AMV reverse transcriptase (TaKaRa) according to manufacturer protocol. RT-qPCR was performed on an ABI StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR premix Ex Taq II (TaKaRa), as previously described [53]. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and the data were normalized against *Pa*18SrRNA.The primers used are listed in Supplementary Table 1. Each PCR trial was performed in triplicate and repeated at least three times.

For *miR-155*-expression analysis, total RNA was prepared and reversed transcribed into first-strand cDNA using the Mir-X miRNA first-strand synthesis kit (Clontech, Mountain View, CA, USA) according to manufacturer instructions. RT-qPCR was performed using the SYBR Advantage qPCR premix kit (Clontech) according to the following protocol: 95 °C for 10 s then 40 cycles of amplification at 95 °C for 50 s and 60 °C for 20 s, followed by melting-curve analysis at 95 °C for 60 s, 55 °C for 30 s, and 95 °C for 30 s. Relative *miR-155*-expression levels were normalized against *U6* small nuclear (sn) RNA, and each PCR trial was performed in triplicate and repeated at least three times. The primers used are listed in Supplementary Table 1.

2.3. miRNA mimics and inhibitors

The miR-155 mimic (MC11056), miR-155 inhibitor (MH11056), and corresponding controls (mirVana miRNA mimic negative control, 4464058; and mirVana miRNA inhibitor negative control, 4464076) were purchased from Ambion, Life Technologies (Austen, TX, USA). Isolated ayu MO/MΦ were transfected with 30 nM of each oligonucleotide for 24 h–72 h using Lipofectamine RNAiMAX reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA), and the expression of *miR-155* was evaluated to determine the best time point of transfection.

2.4. Ayu head-kidney-derived MO/MΦ isolation and V. anguillarum stimulation

Ayu head-kidney-derived MO/MΦ were isolated as previously described [53]. Briefly, head-kidney leukocyte-enriched fractions were obtained by applying dissociated ayu head-kidney cells to a Ficoll (1.077 g/mL; GE Healthcare, Chicago, IL, USA) density gradient. The cells were then seeded in 35-mm dishes at a density of 2×10^7/mL and cultured overnight at 24 °C under 5% CO_2. After washing away the non-adherent cells, attached cells were incubated with complete RPMI 1640 medium (5% fetal bovine serum, 5% ayu serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) and cultured under the same conditions. For *V. anguillarum* stimulation, isolated ayu head-kidney MO/MΦ were infected with live *V. anguillarum* at a multiplicity of infection (MOI) of 10. PBS-stimulated cells were used as controls. Cells were collected at 4, 8, 12, 24, and 48 hpi for RNA extraction, and RT-qPCR was conducted as described.

To evaluate the role of miR-155 on the expression of cytokines and essential signaling adaptors and regulators following *V. anguillarum* stimulation, isolated ayu MO/MΦ were transfected with 30 nM miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before stimulation with *V. anguillarum.* Cells were collected at 4, 8, 12, and 24 hpi, and RT-qPCR was conducted. The primers used are listed in Supplementary Table 1.

2.5. In vitro phagocytosis assay

The phagocytosis activity of ayu MO/MΦ was assessed, as previously described [53]. Logarithmic phase *Escherichia coli* DH5α cells were heat-inactivated and labeled with fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO, USA) according to manufacturer instructions and designated as FITC-DH5α. Isolated ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h prior to incubation with FITC-DH5α (MOI: 10) for 30 min. The cells were thoroughly washed with PBS and incubated with trypan blue (0.4%) to quench the fluorescence outside of the cell membrane. The MO/MΦ were then harvested and resuspended in 500 μL PBS, engulfed bacteria were examined using a MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany), and data were analyzed with MACSQuant analysis software (Miltenyi Biotec). Relative mean fluorescence intensity (MFI) of the miR-155-mimic- or miR-155-inhibitor-transfected group was expressed as fold change relative to the value of the corresponding negative-control-transfected groups (assigned a unit of 100).

2.6. In vitro bacterial-killing assay

Isolated ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before being infected with live *V. anguillarum* at an MOI of 10. The phagocytosis of bacteria was allowed to proceed for 30 min at 24 °C under 5% CO_2. The remaining bacteria were killed using gentamicin (50 μg/mL), and the MO/MΦ were washed with sterile PBS. Each set of transfected MO/MΦ were divided into two groups. One group (the uptake group) was immediately lysed with 1% Triton X-100 solution and plated onto solid thiosulfate-citrate-bile-salts-sucrose (TCBS) agar medium to evaluate bacterial-uptake values. The other group (the kill group) was incubated for an additional 1.5 h prior to lysis and plating on TCBS agar medium. After incubation at 28 °C for 12 h, the CFUs of the plates were calculated. Bacterial survival was determined by dividing the CFUs in the kill group by those in the uptake group. Three independent experiments were performed.

2.7. MO/MΦ polarization assay

To analyze the role of miR-155 on MO/MΦ polarization, LPS-induced M1-type and cAMP-induced M2-type MO/MΦ were prepared according to previously described methods [55]. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before treatment with LPS (50 μg/mL; *E. coli*; Sigma-Aldrich) or a cAMP analog (dibutyryl cAMP; 0.5 mg/mL, Sigma-Aldrich) for 6 h. The RNAiMAX-PBS-only treated group was used as the negative control to exclude the possible toxicity effects of the transfection reagent. Expression levels of the pro-inflammatory cytokines *TNF-α* and *IL-1β* and anti-inflammatory cytokines *TGF-β* and *IL-10* were determined. In addition, the induced nitric oxide synthase (iNOS) (for LPS-induced M1-type MO/MΦ) and arginase activities (for cAMP-induced M2-type MO/MΦ) were also evaluated.

iNOS activity was measured using a nitric oxide synthase assay kit (fluorescence probe method; Beyotime, Shanghai, China) according to manufacturer protocol. Corresponding miRNA-transfected cells were stimulated with LPS for 18 h at 24 °C, with the RNAiMAX-PBS-only treated group used as a negative control. The relative iNOS activity of each group was expressed as fold change relative to the value of the PBS-only treated group. Arginase activity was measured using an arginase activity assay kit (Sigma-Aldrich) according to manufacturer protocol. Corresponding miRNA-transfected cells were stimulated with cAMP for 18 h at 24 °C, with the RNAiMAX-PBS-only treated group used as a negative control. Absorbance was read at 430 nm, and arginase activity (u/L) was calculated according to comparison with urea-standard data.

2.8. Apoptosis assay

Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before infection with *V. anguillarum* at an MOI of 2 for 8 h. The cells were harvested, washed, and labeled with Annexin V-FITC and propidium iodide (PI) for 15 min using a FITC Annexin V apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA). Apoptosis was evaluated using a MACSQuant Analyzer 10 (Miltenyi Biotec) within 30 min of staining, and data were analyzed using MACSQuant analysis software (Miltenyi Biotec).

2.9. Statistical analysis

All data represent the means ± SEM. The statistical analysis of study results was conducted by one-way analysis of variance (ANOVA) using SPSS version 13.0 (SPSS Inc, Chicago, IL, USA). The *p* values $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered statistically significant.

3. Results

3.1. miR-155 tissue distribution and expression following V. anguillarum infection

miR-155 expression was detected in the heart, brain, muscle, intestine, skin, head kidney, liver, gill, and spleen, with the highest level of *miR-155* transcripts observed in the spleen, followed by the gill, liver, and head kidney (Fig. 1A). Upon *V. anguillarum* injection, *miR-155* expression was upregulated in all tested tissues, except the intestine, in a time-dependent manner. *miR-155* levels in the gill were increased at 4 hpi and maintained a similar level until 48 hpi. The highest *miR-155* levels in the head kidney, liver, and spleen were detected at 8 hpi, followed by a subsequent decrease. The *miR-155* levels were decreased

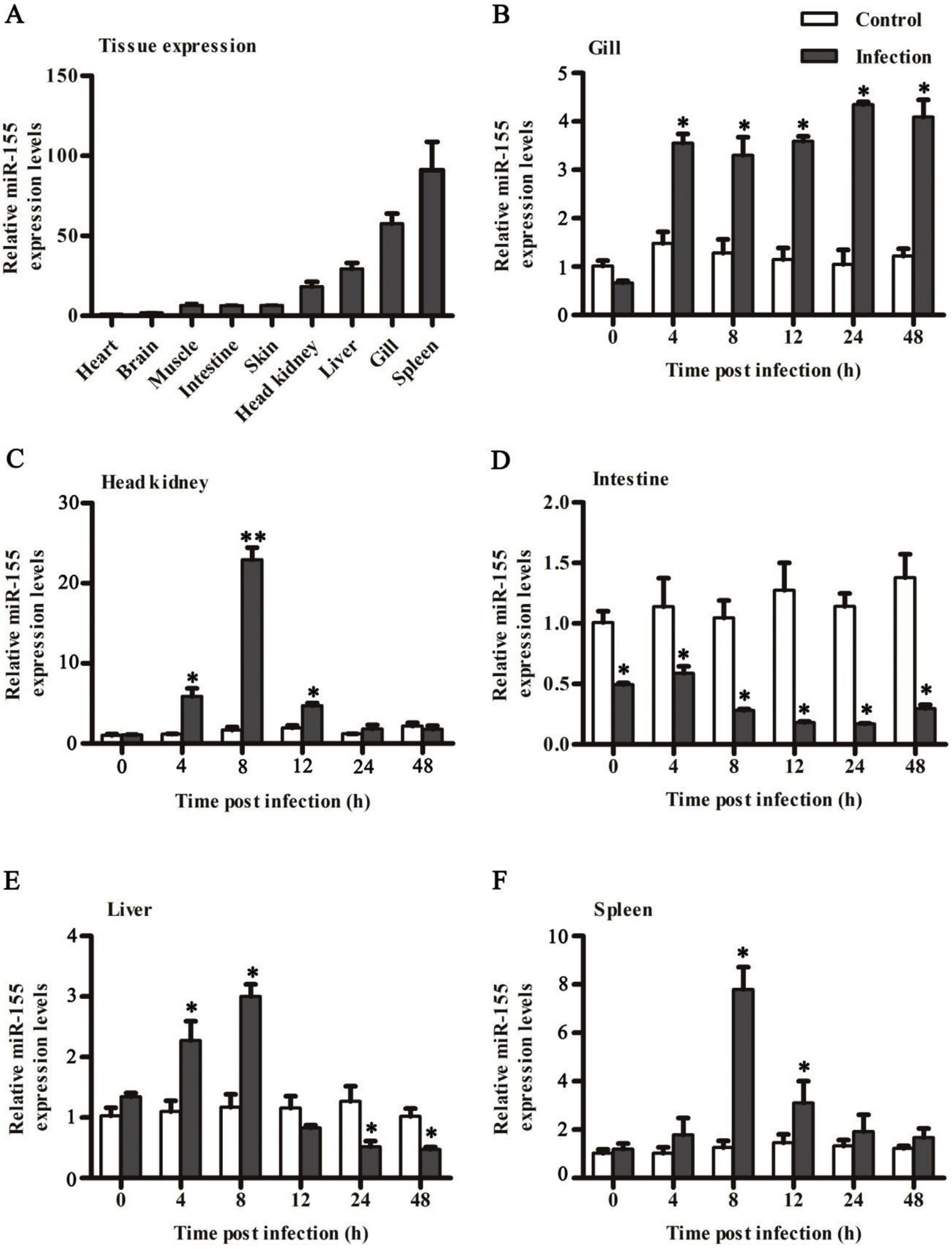

Fig. 1. qPCR analysis of *miR-155*-expression patterns in ayu tissues. (A) Relative *miR-155*-expression levels in healthy ayu tissues (heart, brain, muscle, intestine, skin, head kidney, liver, gill, and spleen) relative to *U6* snRNA levels. Tissue samples were extracted from three fish at each time point. *miR-155* expression in healthy heart was normalized to 1. (B–F) *miR-155*-expression levels in ayu challenged with *V. anguillarum*. Tissues were collected at 0, 4, 8, 12, 24, and 48 hpi, and the relative *miR-155* levels to *U6* snRNA were quantified, with *miR-155* levels in the 0 h PBS-injected group normalized to 1. Data represent the means ± SEM of three replicates. $^{*}p < 0.05$ and $^{**}p < 0.01$, *V. anguillarum*-infected group vs. PBS-injected control.

in the intestine immediately following *V. anguillarum* injection, which might have been due to the prevention of excessive intestinal inflammation (Fig. 1B–F).

3.2. MiR-155 affects cytokine mRNA expression in V. anguillarum-infected ayu MO/MΦ

To evaluate the role of miR-155 in bacteria-induced immune responses, we investigated its expression patterns in ayu MO/MΦ and its regulatory roles on the expression of pro-inflammatory (*IL-1β* and *TNF-α*) and anti-inflammatory cytokines (*IL-10* and *TGF-β*) following *V. anguillarum* infection. We first determined the effects of and optimal time for miR-155-mimic and -inhibitor overexpression. As shown in Fig. 2, transfection of the miR-155 mimic increased *miR-155* expression, whereas miR-155-inhibitor transfection decreased *miR-155* expression, with both peak levels detected at 24-h post-transfection (hpt). Hence, the time point for miR-155 transfection used in the following experiments was 24 hpt.

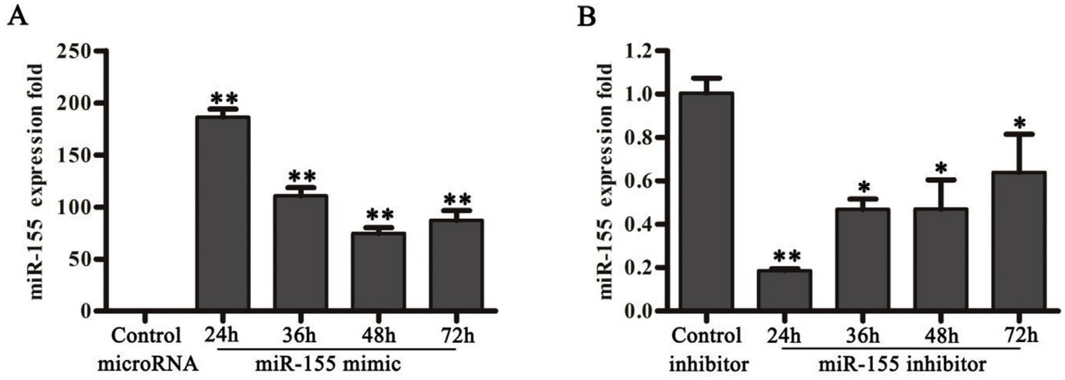

Fig. 2. The effects of and optimal time for miR-155-mimic and -inhibitor overexpression. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls at a final concentration of 30 nM. At 24, 36, 48, and 72 hpt, cells were collected, and miR-155 expression was measured by qRT-PCR and normalized against *U6* snRNA. Levels in the control groups were set to 1. Data represent the means ± SEM of three replicates. $^*p < 0.05$ and $^{**}p < 0.01$.

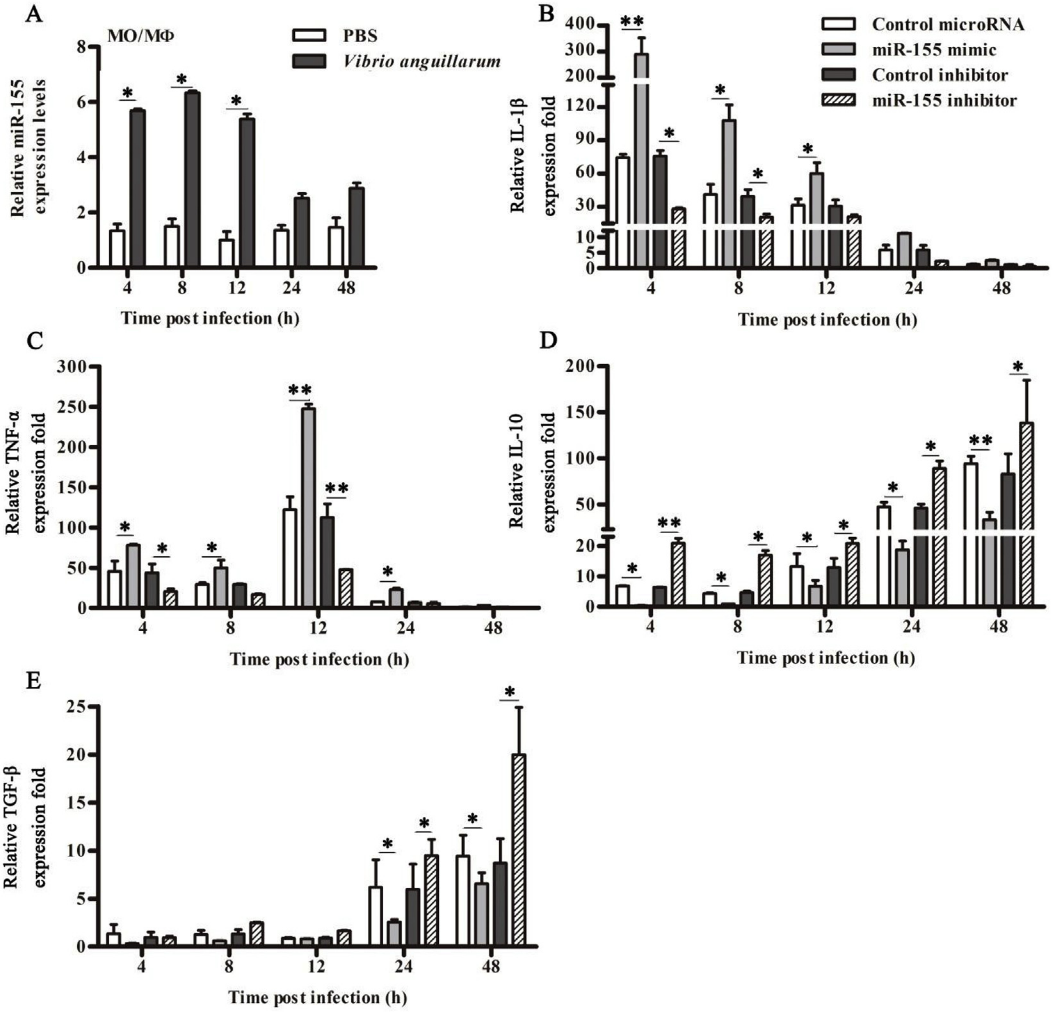

Fig. 3. miR-155 enhances *IL-1β* and *TNF-α* expression and suppresses *IL-10* and *TGF-β* expression in *V. anguillarum*-infected ayu MO/MΦ. (A) *miR-155* expression in *V. anguillarum*-infected ayu MO/MΦ at 4, 8, 12, 24, 48 hpi relative to *U6* snRNA. *miR-155* expression at 4 hpi was normalized to 1. (B–E) Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before *V. anguillarum* infection. Cells were collected at 4, 8, 12, 24, 48 hpi, and relative *IL-1β* (B), *TNF-α* (C), *IL-10* (D), and *TGF-β* (E) mRNA levels were analyzed against *Pa*18S rRNA and normalized against those in the PBS-treated non-infected group. Data represent the means ± SEM of three replicates. $^*p < 0.05$ and $^{**}p < 0.01$.

Assessment of *miR-155*-expression patterns in *V. anguillarum*-infected ayu MO/MΦ revealed substantial upregulation at 4 hpi, with this maintained until 12 hpi (Fig. 3A). At 24 hpi, *miR-155* expression returned to control levels. To explore the role of miR-155 in modulating inflammatory cytokine expression during *V. anguillarum* infection, MO/MΦ were transfected with 30 nM miR-155 mimic, miR-155 inhibitor, or corresponding controls prior to *V. anguillarum* infection for 24 h. The results showed that miR-155 overexpression markedly increased the expression of pro-inflammatory cytokines (*IL-1β* and *TNF-α*; Fig. 3B and C) and decreased the expression of anti-inflammatory cytokines (*IL-10*

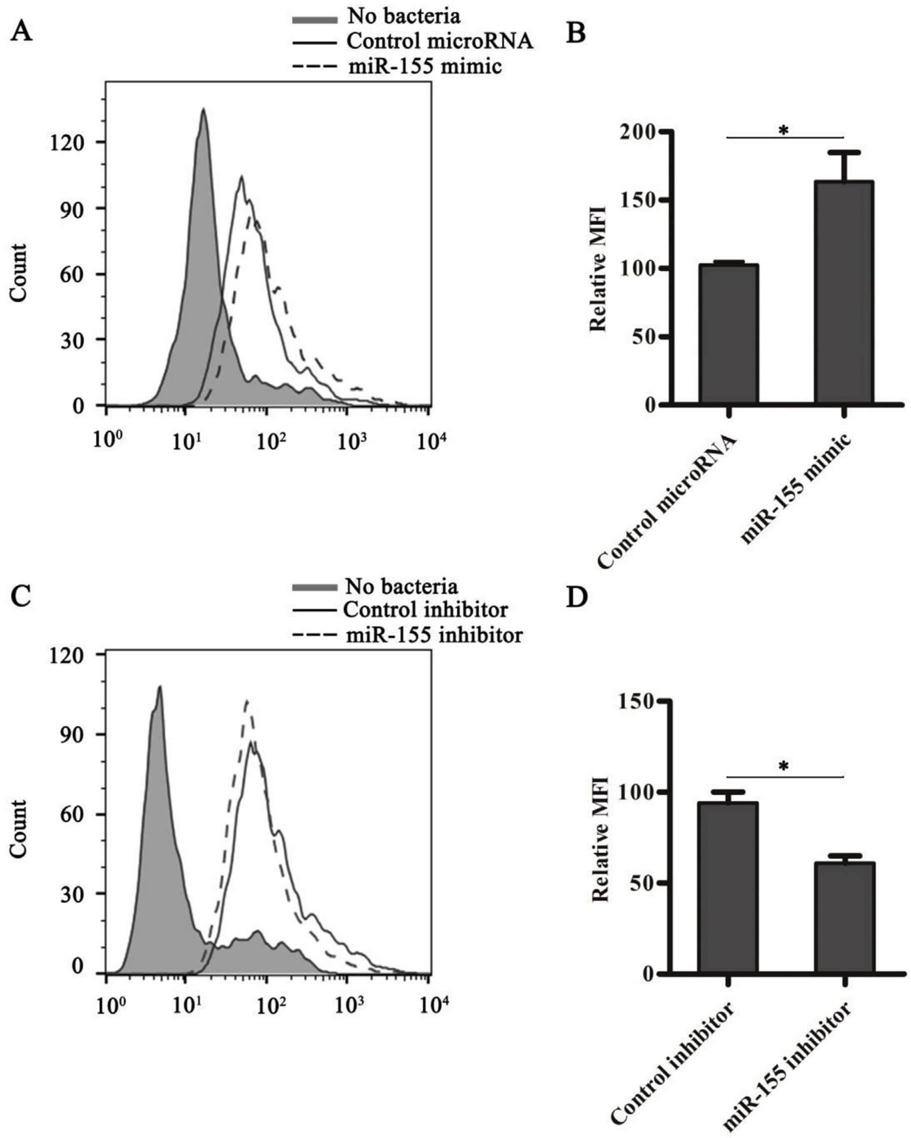

Fig. 4. MiR-155 promotes the phagocytosis activity of ayu MO/MΦ. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h, followed by the addition of FITC-DH5α at an MOI of 10 and incubation for an additional 30 min (A, C) Flow cytometric analysis of the phagocytosis of FITC-DH5α of miR-155-overexpressing MO/MΦ, miR-155-inhibited MO/MΦ, and their corresponding control. (B, D) Relative MFIs of these groups were also analyzed, with values for the control groups assigned a unit of 100. Data represent the means ± SEM of three replicates. $*p < 0.05$ and $**p < 0.01$. MFI, mean fluorescence intensity.

and *TGF-β*; Fig. 3D and E) as compared with levels in the control group. Alternatively, inhibition of miR-155 expression decreased IL-1β and TNF-α levels and increased IL-10 and TGF-β levels (Fig. 3B–E) relative to the control group. These results indicated that miR-155 might exert a pro-inflammatory function in MO/MΦ during *V. anguillarum* infection.

3.3. miR-155 promotes the phagocytosis and bacterial-killing activities of ayu MO/MΦ

As miR-155 might play a pro-inflammatory function in ayu MO/MΦ during *V. anguillarum* infection, we further evaluated its ability to regulate phagocytosis and bacterial-killing activities of ayu MO/MΦ. As shown in Fig. 4, *miR-155* overexpression significantly promoted the phagocytosis of MO/MΦ whereas its inhibition decreased this activity. Additionally, measurement of intracellular *V. anguillarum* CFUs in ayu MO/MΦ showed that the bacterial survival rate in the *miR-155*-overexpression group (24.30%) was lower than that of the control microRNA transfected group (38.95%) (Fig. 5A). Alternatively, inhibition of *miR-155* expression significantly increased the bacterial survival rate (55.01%) relative that observed in the control (Fig. 5B).

3.4. MiR-155 is involved in the regulation of MO/MΦ polarization

Because miR-155 plays important regulatory roles in the immune responses of ayu MO/MΦ, we further evaluated its involvement in regulating LPS-induced M1-type polarization and cAMP-induced M2-type polarization of MO/MΦ. M1-type polarization is characterized by the expression of pro-inflammatory cytokines and production of reactive oxygen species (ROS) and NO [56], whereas M2 type polarization is characterized by the expression of anti-inflammatory cytokines and increased arginase activity [57]. As shown in Fig. 6, LPS treatment markedly increased the expression of the pro-inflammatory cytokines *TNF-α* and *IL-1β*, with these levels even higher in the miR-155-overexpressing group, whereas miR-155 inhibition downregulated these LPS-induced levels (Fig. 6A and B). Additionally, LPS stimulation induced the expression of the anti-inflammatory cytokines *IL-10* and *TGF-β*, with these levels significantly suppressed in miR-155-overexpressing MO/MΦ and enhanced in miR-155-inhibited MO/MΦ (Fig. 6C and D). As an indicator of M1-type polarization, we observed increased iNOS activity in LPS-stimulated MO/MΦ, with this activity even higher in miR-155-overexpressing MO/MΦ, and suppressed in miR-155-inhibited MO/MΦ (Fig. 6E). These results indicated that miR-155 promoted the M1-type polarization of ayu MO/MΦ.

By contrast, cAMP treatment significantly increased *IL-10* and *TGF-β*

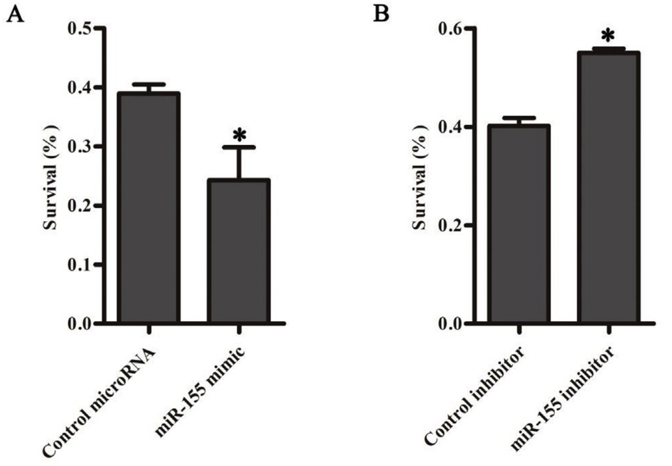

Fig. 5. miR-155 promotes the bacterial-killing activity of ayu MO/MΦ. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before infection with *V. anguillarum*. *V. anguillarum* viability was examined using a CFU assay. (A, B) Histograms showing the relative survival rates of *V. anguillarum* in the *miR-155*-overexpressing group, *miR-155*-inhibited, and their respective control groups. Data represent the means ± SEM of three replicates. $^{*}p < 0.05$ and $^{**}p < 0.01$.

expression and the arginase activity of MO/MΦ, all of which were suppressed by miR-155 overexpression but induced to an even greater degree by miR-155 inhibition (Fig. 7C–E). Additionally, miR-155 overexpression induced *TNF-α* and *IL-1β* expression following cAMP stimulation, whereas *TNF-α* mRNA levels were decreased in *miR-155*-inhibited MO/MΦ (Fig. 7A and B).

3.5. MiR-155 augments the V. anguillarum-induced apoptosis of ayu MO/MΦ

Because miR-155 promotes pro-inflammatory and antibacterial processes associated with MO/MΦ during *V. anguillarum* infection, and given that excessive inflammatory responses and the accumulation of ROS and inflammatory cytokines can affect cell apoptosis [58–60], we evaluated the involvement of miR-155 in regulating *V. anguillarum*-induced apoptosis of ayu MO/MΦ. We found that the apoptosis levels in control-miRNA- and control-miRNA-inhibitor-transfected MO/MΦ were 46.39% ± 3.94% and 46.47 ± 4.91%, respectively, following infection with *V. anguillarum*. However, miR-155-overexpressing MO/MΦ exhibited increased apoptosis levels (58.36 ± 1.69%) while miR-155-inhibited MO/MΦ showed significantly lower levels of apoptosis (27.30 ± 2.77%) (Fig. 8).

PI, propidium iodide; Van, *V. anguillarum* infection.

4. Discussion

miR-155 represents an important miRNA involved in regulating numerous cellular and biological processes. Previous studies in mammals demonstrated miR-155 as a common target of a broad range of inflammatory mediators in macrophages [61], with macrophage-related inflammatory responses, such as regulation of phagocytosis, autophagy, lipid uptake, and polarization, enhanced or suppressed by miR-155 depending on the cell types or stimulators [26,27,62–64]. Manuel et al. reported that miR-155 inhibits DNA-damage-induced apoptosis, whereas Devramet et al. indicated that miR-155 induces *Mycobacterium bovis* BCG-mediated apoptosis [65,66]. We speculate that such discrepancies might be a consequence of differences in stimulation and activation of associated signaling pathways. Overall, previous findings suggest that miR-155 exerts multifunctional and bidirectional regulatory functions, depending on the specific type of macrophage and signaling activated.

Despite thorough investigations in mammals, studies of the regulatory roles of miR-155 in teleosts are limited. In the present study, we investigated the role of miR-155 on the immune-related responses and apoptosis of ayu MO/MΦ during *V. anguillarum* infection. We detected the highest expression of *miR-115* in the spleen, followed by the gill, liver, and head kidney. Upon *V. anguillarum* infection, *miR-155* levels were upregulated in all immune tissues, except the intestine, in a time-dependent manner, with these results suggesting the potential involvement of miR-115 in ayu immune responses against pathogen infection.

As important components of the immune system, MO/MΦ are critical for pathogen clearance and involved in innate and adaptive immunity. Therefore, we determined the regulatory role of miR-155 in the immune functions of ayu MO/MΦ. We found that *miR-155* levels were upregulated in MO/MΦ during *V. anguillarum* infection, with miR-155 overexpression promoting the expression of pro-inflammatory cytokines and suppressing that of anti-inflammatory cytokines, suggesting miR-155 as a positive regulator of MO/MΦ-specific immune responses. Furthermore, phagocytosis and bacterial-killing assays demonstrated significant promotion of phagocytosis activity in the presence of FITC-DH5α, and that the survival rate of *V. anguillarum* was decreased by ~14.55% in miR-155-overexpressing MO/MΦ.

Polarization in response to microenvironmental signals constitutes another indicator of the immune state of ayu MO/MΦ [67,68]. In the present study, we observed that miR-155 promoted LPS-induced M1-type polarization characterized by the increased expression of pro-inflammatory cytokines, decreased expression of anti-inflammatory cytokines, and enhanced iNOS activity. Alternatively, cAMP-induced M2-type polarization was inhibited by miR-155 according to the decreased expression of proinflammatory cytokines, increased expression of anti-inflammatory cytokines, and enhanced arginase activity. Apoptosis represents a method of resolving acute inflammatory response of immune cells, with apoptotic activity capable of being induced by accumulated ROS. Because miR-155 could aggravate inflammation, and given that ROS production accompanies bacterial-killing processes associated with MO/MΦ, we hypothesized that miR-155-induced inflammation might result in MO/MΦ apoptosis. Our experimental results confirmed that miR-155 overexpression increased apoptosis, whereas miR-155 silencing significantly decreased the percentage of apoptotic cells relative to their respective controls.

miRNAs exert their function by binding to the 3′ UTRs of target genes to degrade mRNA or inhibit translation [69,70]. To further investigate miR-155 targets involved in pro-inflammatory functions, we preliminary screened possible targets of miR-155 in *V. anguillarum*-infected MO/MΦ. Because TLRs represent one of the most important signaling pathways involved in bacteria-induced immune responses, we examined the role of miR-155 on the expression of essential TLR-signaling adaptors. Our results showed that except for *TBK1*, the expression of *MyD88*, *TRIF*, and *TAK1* were upregulated in miR-155-overexpressing MO/MΦ (Supplementary Figs. 1A, B, D, and E). This agreed with the above observed pro-inflammatory effects associated with miR-155. However, miR-155 showed no regulatory effects on the expression of *MAVS* (an adaptor for RLR signaling; Supplementary Fig. 1C), although MAVS has also been shown to be involved in both antiviral and antibacterial immune responses [71–73]. As miR-155 promotes pro-inflammatory responses, we hypothesized a possible role in degrading the mRNA of molecules involved in negatively regulating TLR signaling. Previous studies using mammalian models reported SOCS1 as a negative regulator of TLR signaling, and that B cell lymphoma 6 (Bcl6) inhibited the acute inflammatory response of macrophages and occasionally downregulated apoptosis [74–78]. Moreover, SOCS1 and Bcl6 are reported targets of miR-155 in mammals [79,80]. In the present study, we showed that *SOCS1* expression was suppressed in miR-155-overexpressing MO/MΦ and induced in *miR-155*-silenced cells (Supplementary Fig. 1F), whereas no significant difference in *Bcl6*

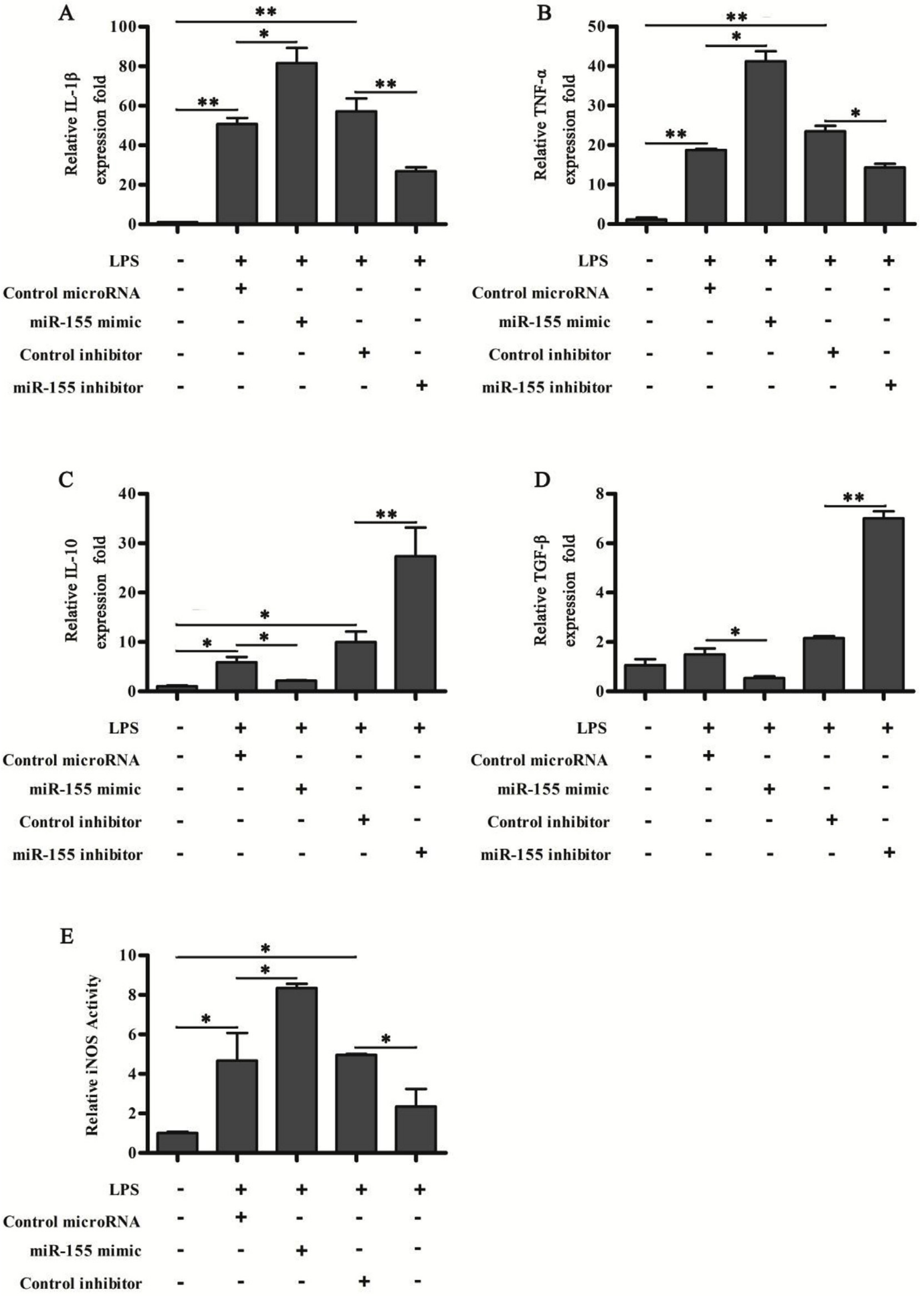

Fig. 6. miR-155 promotes the M1-type polarization of ayu MO/MΦ. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h prior to stimulation with LPS for 6 h (to evaluate cytokine expression) or 18 h (to evaluate iNOS activity). The RNAiMAX-PBS-only treated group was used as a negative control (first lane). (A, B, E) LPS treatment markedly increased *TNF-α* and *IL-1β* expression, as well as iNOS activity, in ayu MO/MΦ, with these levels even higher in miR-155-overexpressing MO/MΦ but suppressed in miR-155-inhibited MO/MΦ. (C, D) *IL-10* and *TGF-β* expression was induced by LPS treatment but suppressed; however, in miR-155-overexpressing MO/MΦ and enhanced in miR-155-inhibited MO/MΦ. Data represent the means ± SEM of three replicates. $*p < 0.05$ and $**p < 0.01$.

expression was observed in either group relative to their respective controls (Supplementary Fig. 1G). These results suggested that SOCS1 might serve as a possible target of miR-155 in ayu MO/MΦ, and the discrepancy between the role of miR155 on Bcl 6 in mammals and ayu maybe due to functional differentiation of Bcl6 during evolution, although further investigation is required to verify this finding.

The SOCS family includes eight members (SOCS1–7 and cytokine inducible SH2-containing protein) in mammals [81]. Among SOCS

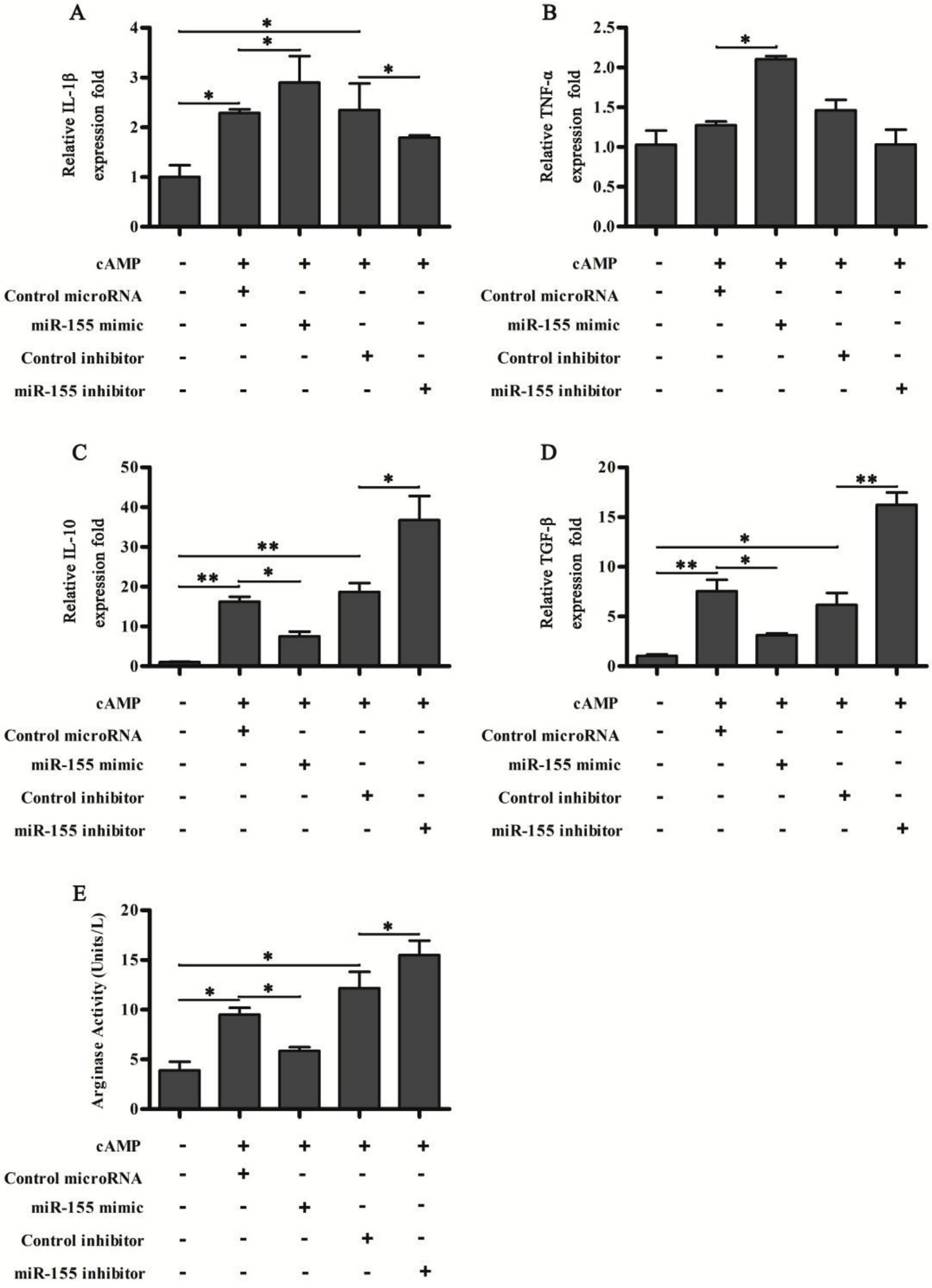

Fig. 7. miR-155 inhibits the M2-type polarization of ayu MO/MΦ. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before stimulation with cAMP for 6 h (to evaluate cytokine expression) or 18 h (to evaluate arginase activity). The RNAiMAX-PBS-only treated group was used as a negative control (first lane). (A, B) miR-155 overexpression induced *TNF-α* and *IL-1β* expression following cAMP stimulation, whereas *TNF-α* mRNA levels were decreased by miR-155 inhibition. (C–E) cAMP treatment significantly increased *IL-10* and *TGF-β* expression and arginase activity in MO/MΦ, all of which were suppressed by miR-155 overexpression but enhanced to an even greater degree by miR-155 inhibition. Data represent the means ± SEM of three replicates. $*p < 0.05$ and $**p < 0.01$.

proteins, SOCS1 represents the most important regulator of innate and adaptive immunity according to its ability to negatively regulate a broad range of immune-relevant signaling pathways, such as TLR, NF-κB, JNK, p38, and interferon signaling, with the most classic target of SOCS1 being the JAK/STAT pathway [74,81–84]. Additionally, SOCS1 reportedly promotes M2-type polarization and inhibits M1-type polarization of macrophages and promotes suppression of apoptosis by regulating JAK or ERK1/2 activation [85–87]. Moreover, miR-155–SOCS1 interactions in mammals is involved in processes associated with apoptosis [88,89]. These findings support our hypothesis that miR-155 targets *SOCS1* to promote the pro-inflammatory functions and apoptosis of *V. anguillarum*-infected ayu MO/MΦ.

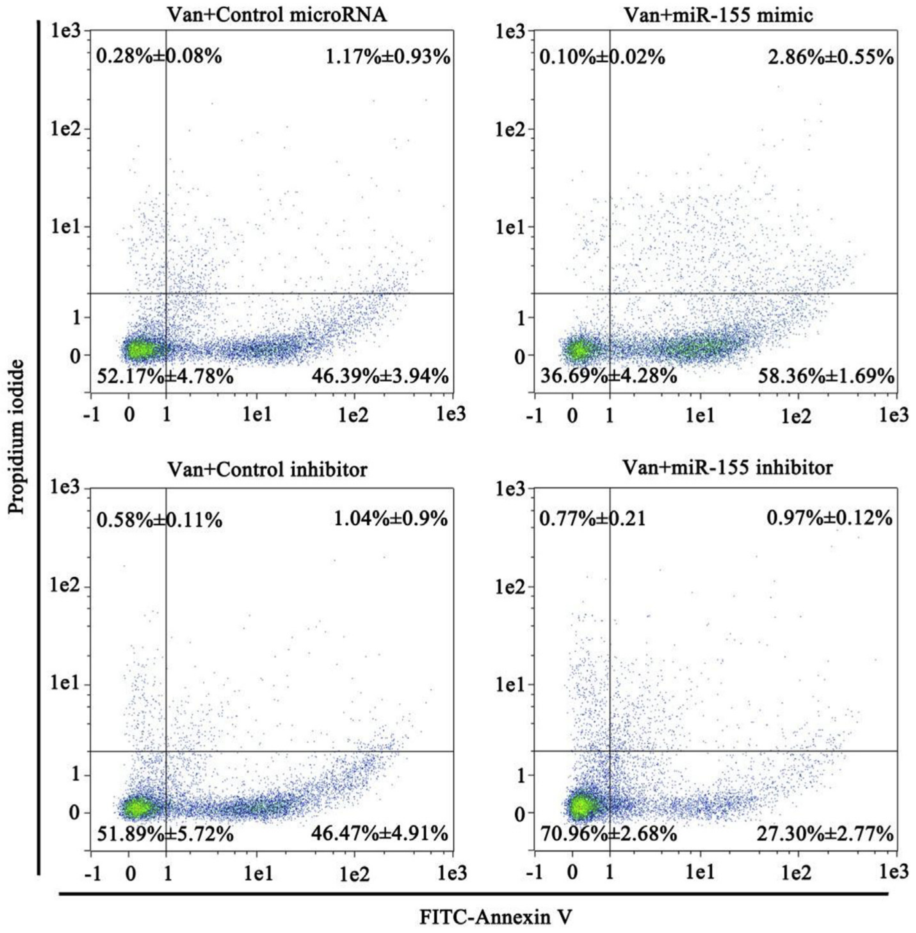

Fig. 8. miR-155 augments *V. anguillarum*-induced apoptosis of ayu MO/MΦ. Ayu MO/MΦ were transfected with miR-155 mimic, miR-155 inhibitor, or corresponding controls for 24 h before infection with *V. anguillarum* at an MOI of 2 for 8 h. Cells were harvested and labeled with Annexin V-FITC and PI, with levels of apoptosis (Annexin V^+PI^-) analyzed by flow cytometry. Data represent the means ± SEM of three replicates.

In summary, we described the roles of miR-155 in promoting the proinflammatory functions of *V. anguillarum*-infected MO/MΦ. Our findings showed that upregulated levels of miR-155 promoted proinflammatory cytokine expression while suppressing that of anti-inflammatory cytokines and promoted the phagocytosis and bacterial-killing functions of ayu MO/MΦ. Moreover, miR-155 augmented LPS-induced M1-type polarization and inhibited cAMP-induced M2-type polarization, suggesting a pro-inflammatory role of miR-155 in ayu MO/MΦ. Furthermore, miR-155 upregulation increased *V. anguillarum*-infected MO/MΦ apoptosis potentially caused by excessive inflammation or ROS accumulation via bacterial killing, and we identified *SOCS1* as a possible miR-155 target involved in regulating ayu MO/MΦ functions. Our results offer critical insight into the immunogenic role of miR-155 in teleosts; however, the precise mechanisms associated with this role require further characterization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.030.

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