The inducible microRNA-203 in fish represses the inflammatory responses to Gram-negative bacteria by targeting IL-1 receptor-associated kinase 4

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Innate immune responses are the first defense against pathogenic invaders. Activation and termination of these immune responses are regulated by several mechanisms. MicroRNAs (miRNAs), a group of small non-coding RNAs, have been implicated in the regulation of a spectrum of both physiological and pathological conditions, including immune responses. Although the immune regulatory miRNA networks in higher vertebrates have been well described, regulation of these responses in fish species is poorly understood. In the present study, we investigated the role of the miRNA miR-203 involved in inflammatory responses in Miiuy croaker (Miichthys miiuy). We found that the Gram-negative bacterium Vibrio anguillarum and lipopolysaccharide significantly up-regulated host miR-203 expression. The increased miR-203 expression suppressed the production of inflammatory cytokines and thereby prevented mounting of a full immune response. Mechanistically, we identified and validated IL-1 receptor-associated kinase 4 (IRAK4) as a target of miR-203. We observed that miR-203 post-transcriptionally controls IRAK4 expression and thereby inhibits the activation of nuclear factor κB (NF-κB) signaling. In summary, our findings reveal that miR-203 in fish is a critical suppressor of innate immune responses to bacterial infection by suppressing a feedback to IRAK4–NF-κB-mediated signaling.

Innate immunity is the primary defense mechanism that recognizes pathogenic microorganisms by various conserved pattern recognition receptors. Toll-like receptors (TLRs),1 as one of the well-documented receptors, detect pathogen-associated molecular patterns and then initiate the subsequent signaling events to defend against infectious pathogens, resulting in a core set of stereotyped response (1). When TLRs (except TLR3) bind to TLR ligands, they recruit the adaptor molecule MyD88 to mediate the MyD88-dependent signaling cascade (2–4). It is well documented that recruitment of adaptor kinases is the prime factor for initiation of the TLR signaling cascade. IL-1 receptor-associated kinase 4 (IRAK4), known as the pivotal adaptor kinase used by almost all TLR signaling, could be recruited to MyD88, forming the MyD88-IRAK4-IRAK2/1 receptor complex that further activates TNFR-associated factor 6 (TRAF6) (5, 6). Afterward, the IRAK-TRAF6 complex dissociates from the receptor complex to interact with TAK1 (7). The activation of TAK1 eventually leads to the activation of NF-κB signaling (8), resulting in transcription of various inflammatory genes, including TNF-α, IL-6, and IL-8, to sense and react to infection. In contrast, dysregulation of this process is detrimental to the host, leading to inflammatory damage and autoimmune diseases. Thus, TLR-mediated innate immune response must be tightly regulated by detailed mechanisms.

Recent studies have proposed a series of regulatory controllers involved in regulating TLR signaling cascades at several layers, including TLR molecules, TLR-related signaling molecules, and TLR-triggered transcription factors and inflammatory genes. Previous studies suggested that Rab7b could negatively regulate TLR4 signaling by direct degradation of TLR4 (9). Phosphatase SHP-1 has been reported to negatively regulate TLR-mediated production of proinflammatory cytokines by interacting with and inhibiting the kinase IRAK1 (10). Additionally, anti-inflammatory cytokines, such as IL-10, could also be the effective suppressors to control TLR signaling activity to avoid excessive inflammation (11). Although much of the focus has been on the study of protein regulators in the TLR signaling pathway, recent evidence has pointed to the potential regulatory role of microRNA in managing this signaling pathway.

MicroRNAs (miRNAs) are small and single-stranded non-coding RNA sequence (21–24 nucleotides) emerging recently as translational repressors of gene transcripts by binding to their 3'-UTRs, resulting in target transcript degradation or protein translation inhibition (12, 13). In mammals, as many as 60% of all protein-coding genes have been documented to be regulated by miRNAs (14). miRNAs act as vital and versatile modulators that regulate the intricate networks controlling fundamental biological processes, including growth, development, proliferation, apoptosis, and immune response (14–16). In the field of inflammation, miRNAs have attracted tremendous interest for their ability to modify strength and timing in TLR-mediated signaling responses. To date, a series of miRNAs have been documented to regulate the different levels of the cascade.

1 To whom correspondence should be addressed. E-mail: tianjunxu@163.com.
2 The abbreviations used are: TLR, Toll-like receptor; IRAK, IL-1 receptor-associated kinase; TRAF6, TNFR-associated factor 6; miRNA or miR, microRNA; LPS, lipopolysaccharide; qPCR, quantitative PCR; EGFP, enhanced GFP; si-Ctrl, scrambled control RNA; si-IRAK4, IRAK4-specific siRNA; Ctrl, control mimics; miR-203-i and Ctrl-i, miR-203 and control inhibitor, respectively.

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This article contains Tables S1 and S2.
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reaction. At the level of regulating TLR expression, the let-7 miRNA family, including let-7e and let-7i, have been reported to modulate TLR4, and microRNA (miR)-19a/b could reduce TLR2 level, thereby inhibiting the production of TLR2-triggered cytokines (17). Although these miRNAs eliminate TLR expression, it is more effective to regulate TLR-related signaling molecules by miRNAs. In higher vertebrates, several downstream TLR-related signaling adaptor proteins are identified as strong miRNA targets. MyD88 has been identified as an miR-155 target in both mice and humans (18). IRAK1 and TRAF6 have also been indicated as targets for miR-146a, which was later confirmed to target IRAK2 (19). In addition, IRAK4 has been identified and validated as a target of miR-132/-212 in monocytes (20). However, the underlying regulatory mechanisms of miRNAs in inflammation upon pathogen infection are much less well studied in fish due to the limitations of research materials and appropriate methods.

Fish, having broad roles in terms of ecology and food production, have been consistently threatened by pathogenic microorganisms, of which Gram-negative bacteria is a major group. Diseases caused by Gram-negative bacteria infection have been the focus of studies because of their widespread, high incidence, and great harm. Recently, the development and aquaculture of the miuy croaker (Miichthys miiuy) has been hindered by vibriosis, leading to the high mortality and severe economic losses (21). *Vibrio anguillarum* is a Gram-negative, comma-shaped rod bacterium, which belongs to the family Vibrionaceae. *V. anguillarum* is an important causative agent of vibriosis, which is a deadly hemorrhagic septicemic disease affecting various fish (22). In the present study, the host regulation mechanism of miRNAs in inflammatory responses is documented in miuy croaker upon *V. anguillarum* infection. We found that treatment with *V. anguillarum*, as well as lipopolysaccharide (LPS) significantly up-regulated miR-203 expression in miuy croaker. Overexpression of miR-203 in LPS-treated macrophages could markedly reduce the production of anti-inflammatory genes. Digging deeper, we demonstrated that miR-203 inhibited the expression of IRAK4 and thus suppressed the activation of NF-κB signaling, thereby attenuating inflammatory response and avoiding excessive inflammation. To the best of our knowledge, this is the first work to show the role of miRNAs in modulating IRAK–NF-κB–mediated inflammatory signaling in fish species and indicates that miR-203 is a candidate modulator involved in inflammation resolution.

Results

miR-203 expression is up-regulated upon Gram-negative bacterial infection

miRNAs have been recently reported as new regulators implicated in the regulation of diverse biological processes by post-transcriptional modulating gene expression. To investigate whether the expression of miRNAs might be regulated upon *V. anguillarum* infection, we examined the miRNA expression profiles in miuy croaker spleen and performed a small RNA deep-sequencing analysis (data not shown). The deep-sequencing data revealed that a series of miRNAs was up-regulated upon *V. anguillarum* infection, with miR-203 being one of the significantly increased miRNAs. To validate the expression profiles of miR-203 upon pathogen stimulation, we determined the expression of miR-203 in *V. anguillarum*-infected miuy croaker liver and spleen samples by real-time qPCR. The expression of miR-203 in both bacterially infected liver and spleen was up-regulated compared with uninfected counterparts (Fig. 1, A and B). To validate the expression of miR-203, we also determined its levels in vivo and in vitro under conditions of stimulation with ultrapure LPS, the endotoxin of Gram-negative bacteria. As shown in Fig. 1C, miR-203 expression was rapidly increased and reached a peak at 6 h in LPS-stimulated macrophages nearly 8-fold higher than that in untreated cells. In agreement with the above results, LPS also exhibited an activating effect on miR-203 expression in LPS-treated miuy croaker kidney sample (Fig. 1D). These findings demonstrated that miR-203 expression could be up-regulated by *V. anguillarum* infection as well as LPS stimulation, indicating its important role in the infectious process of Gram-negative bacterium.

miR-203 represses LPS-induced inflammatory responses

To determine the potential role of miR-203 in bacterial infection, we examined the effect of miR-203 on LPS-induced inflammatory cytokine gene expression in miuy croaker macrophages. Using our previously reported methods (23), we first formed useful experimental models to overexpress or underexpress miR-203 in miuy croaker macrophages. As shown in Fig. 2 (A and B), transfection of miR-203 mimics into miuy croaker macrophages increased miR-203 expression, whereas miR-203 inhibitors decreased its expression. Afterward, we probed the contribution of miR-203 to inflammatory cytokine gene expression upon LPS stimulation. Miuy croaker macrophages were thus transfected with chemically synthesized miR-203 mimics and inhibitors for up to 24 h and then stimulated with LPS for several time periods. As shown in Fig. 2C, miR-203

**Figure 1. The expression profiles of miR-203 detected by real-time qPCR.** Shown are expression profiles of miR-203 after *V. anguillarum* infection in liver (A) and spleen (B) samples and expression profiles of miR-203 after LPS stimulation in miuy croaker macrophages (C) and kidney samples (D). The data were normalized to 5.8S rRNA. Results are standardized to 1 in control untreated cells. In agreement with the above results, LPS also exhibited an activating effect on miR-203 expression in LPS-treated miuy croaker kidney sample (Fig. 1D). These findings demonstrated that miR-203 expression could be up-regulated by *V. anguillarum* infection as well as LPS stimulation, indicating its important role in the infectious process of Gram-negative bacterium.
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To identify the molecular target of miR-203, we first searched for predicted miR-203 targets using bioinformatics tools (Table S1). Among the possible targets of miR-203, we focused on IRAK4, which has been repeatedly demonstrated to be a critical player involved in TLR-dependent immune response. To dissect the possibility that IRAK4 is the target gene for miR-203, we constructed reporter plasmids by cloning 3′-UTR of miiuy croaker IRAK4 into the pmirGLO luciferase reporter within the mutation at miR-203 binding site as a control and then transfected with these plasmids into HEK293 cells for Dual-Luciferase reporter assays. As shown in Fig. 3A, transfection of miR-203 mimics inhibited the luciferase activity by 43% compared with control mimics, whereas both the control and miR-203 mimics showed no effect on activity of luciferase reporter containing the mutated IRAK4 3′-UTR. The significantly down-regulation mechanism was further investigated by using miR-203 inhibitors, revealing that inhibition of luciferase activity by miR-203 mimics was attenuated after cotransfection with miR-203 inhibitors (Fig. 3B). In addition, the concentration and time gradient experiments were conducted, and the result indicated that miR-203 could reduce the level of the luciferase reporter containing the wildtype IRAK4 3′-UTR, and the down-regulation mechanism showed dose-dependent and time-dependent characteristics (Fig. 3, C and D).

Because the miRNA-processing system is conserved from invertebrates to vertebrates (24, 25), we constructed pre-miR-203 plasmid and then transfected it into HEK293 cells for in vitro expression. After transfection of pre-miR-203 plasmids into HEK293 cells, we observed that overexpression of pre-
miR-203 could be sufficient to decrease luciferase activity compared with control plasmid, whereas no change of luciferase was observed in cells transfected with mutated-type IRAK4 3’-UTR in the presence of pre-miR-203 (Fig. 3E). Moreover, pre-miR-203 was found to down-regulate GFP gene expression when the 3’-UTR of IRAK4 was cloned into pEGFP-C1 vector in HEK293 cells (Fig. 3F). Consistent with miR-203, pre-miR-203 also showed a dose-dependent effect on inhibition of luciferase activity at both 24 and 48 h post-transfection (Fig. 3G). In summary, our data adequately revealed that IRAK4 is a target of miR-203.

**miR-203 inhibits the abundance of IRAK4 at both mRNA and protein levels**

Given that miRNAs modulate target gene expression through mRNA degradation or translation inhibition, we next investigated the function of miR-203 in the regulation of IRAK4 expression. To this end, we determined the expression levels of IRAK4 in miuoy croaker macrophages treated with miR-203 mimics or inhibitors. As shown in Fig. 4A, transfection of miR-203 mimics decreased the protein level of IRAK4 in a time-dependent manner, and the inhibition effect appeared more significant at 48 h post-transfection in miuoy croaker macrophages. In contrast, miR-203 inhibitors increased IRAK4 protein level compared with control inhibitors, and the activating effect also presented in a time-dependent manner (Fig. 4B). To assess whether miR-203 could also regulate the expression of IRAK4 at mRNA levels, we transfected with miR-203 mimics, miR-203 inhibitors, or negative controls into miuoy croaker macrophages for 24 h and then stimulated with LPS for 6 and 12 h. As shown in Fig. 4C, miR-203 mimics could reduce the mRNA expression of IRAK4, and the inhibition effect appeared more significant at 6 h post-stimulation. In contrast, miR-203 inhibitors raised the mRNA expression of IRAK4, which significantly up-regulated its expression at 6 h post-stimulation (Fig. 4D). The results suggested that miR-203 could regulate IRAK4 expression at both the protein and mRNA levels.

Additionally, to further examine the effect of miR-203 on the regulation of IRAK4 expression, we constructed IRAK4 expression plasmid that contains the full-length CDS region and 3’-UTR of miuoy croaker IRAK4 and transfected it into HEK293 cells. As shown in Fig. 4E, overexpression of miR-203 exerted a significant inhibitory effect on the mRNA level of IRAK4 as well as on its protein level in a dose-dependent manner. Consistent with miR-203, pre-miR-203 also reduces the expression of IRAK4 at both mRNA and protein levels (Fig. 4F). Taken together, these results demonstrated that IRAK4 is a direct target of miR-203, which could negatively regulate IRAK4 expression at both mRNA and protein levels.

**miR-203 inhibits IRAK4-mediated NF-κB signaling**

IRAK4 is known as the pivotal adaptor kinase and has been demonstrated to be an important player in TLR-dependent immune responses in mammals (5, 6). To explore the function of IRAK4 in miuoy croaker, we first examined the expression levels of IRAK4 upon pathogen infection. The mRNA expres-

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**Figure 3. miR-203 targets miuoy croaker IRAK4.** A, schematic diagram of the predicted target sites of miR-203 in 3’-UTR of IRAK4. HEK293 cells were transfected with miR-203 or Ctrl, along with the wildtype of IRAK4 3’-UTR (WT) or the mutant of IRAK4 3’-UTR (MT) for 24 h, and the luciferase activity was determined. B, HEK293 cells were cotransfected with IRAK4 3’-UTR (WT), together with Ctrl, miR-203, Ctrl-i, or miR-203-i for 24 h, and the luciferase activity was determined. For each transfection, the total amount of oligonucleotides was controlled and normalized (final concentration, 100 nM). C, the miR-203 (0, 30, 60, and 90 nM) together with Ctrl (90, 60, 30, and 0 nM) were cotransfected into HEK293 cells for 24 h, and then the luciferase activity was determined. D, the time gradient was conducted for transfection. E, HEK293 cells were transfected with pre-miR-203, along with the wildtype of IRAK4 3’-UTR (WT) or the mutant of IRAK4 3’-UTR (MT) for 24 h, and the luciferase activity was determined. F, HEK293 cells were cotransfected with pEGFP-IRAK4 3’-UTR or mutant, together with pre-miR-203 or Ctrl. At 48 h post-transfection, the fluorescence intensity was evaluated. G, HEK293 cells were transfected with pre-miR-203 3’-UTR, together with the concentration gradient of pre-miR-203. After 24 or 48 h, the luciferase activity was determined. Luciferase activity was normalized to Renilla luciferase activity. Data are presented as the means ± S.E. (error bars) from at least three independent triplicate experiments. **, p < 0.01; *, p < 0.05 versus the controls.
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**Figure 4. miR-203 suppresses the expression of IRAK4 at post-transcriptional level.** A and B, the miuy croaker macrophages were transfected with miR-203 or Ctrl (A) and miR-203-i or Ctrl-i (B). After 24- or 48-h transfection, the protein levels of IRAK4 were determined by Western blotting. C and D, the miuy croaker macrophages were transfected with miR-203 or Ctrl (C) and miR-203-i or Ctrl-i (D) for 24 h and then stimulated with LPS for 6 and 12 h, respectively. The expression levels of IRAK4 were measured by real-time qPCR and normalized to β-actin. E and F, HEK293 cells were cotransfected with IRAK4 expression plasmid, which contains the full-length CDS region and 3′-UTR of miuy croaker IRAK4, along with Ctrl or miR-203 (E) and pre-miR-203 or pcDNA6.2 (F) in a concentrate gradient manner, and Ctrl (E) and pcDNA6.2 (F) were used to control for the same amount of molecules for transfections. After 48 h, IRAK4 protein and mRNA levels were determined by Western blotting and real-time qPCR, respectively. Data are presented as the means ± S.E. (error bars) from three independent triplicate experiments. **, *p < 0.01; *, p < 0.05 versus the controls.

Expression profiles of IRAK4 in liver, spleen, and kidney after *V. anguillarum* infection were analyzed. As shown in Fig. 5A, 5B, C, and D, Gram-negative bacterium *V. anguillarum* could significantly up-regulate IRAK4 mRNA expression in these immune tissues. Consistent with this, purified LPS also exhibited an activating effect on IRAK4 mRNA expression in liver and kidney samples (Fig. 5B). Moreover, the expression profiles of IRAK4 were detected in LPS-treated miuy croaker macrophages, and the results also showed that IRAK4 mRNA expression was up-regulated upon LPS stimulation (Fig. 5C). These results indicated that IRAK4 could be involved in immune response. Based on this result, we then further explored the regulation role of miuy croaker IRAK4. To this end, we transfected of miiuy croaker IRAK4. To this end, we transfected with miiuy croaker IRAK4 expression plasmid, together with reporter gene NF-κB, IL-8, and IL-1β into HeLa cells. After a 24-h transfection, HeLa cells were stimulated with LPS for another 6 h and then measured for Dual-Luciferase reporter assays. As shown in Fig. 6A, miR-203 mimics sharply suppressed the activation of the NF-κB reporter gene, whereas the inhibitory effect was weakened by cotransfection with the miR-203 inhibitors. The results indicated that upon LPS stimulation, miR-203 played a negative regulation role in NF-κB activation. Consistent with this, miR-203 also showed the negative effect on reporter genes IL-8 and IL-1β upon LPS stimulation (Fig. 6A). Moreover, pre-miR-203 plasmid was used to further verify these results. As shown in Fig. 6B, pre-miR-203 showed the attenuated effect on NF-κB, IL-8, and IL-1β reporter genes, and the inhibitory effect appeared more significant at 24 h post-transfection (Fig. 6C). Collectively, these data sufficiently demonstrated that miR-203 could negatively regulate IRAK4–NF-κB signaling in a manner dependent on modulating IRAK4 upon LPS stimulation.

**Knockdown of IRAK4 inhibits the inflammatory responses**

To confirm the contribution of IRAK4 on the regulation of inflammatory cytokines, we then examined the expression of inflammatory cytokines in LPS-treated macrophages. After transfection with IRAK4-specific siRNA (si-IRAK4) into miuy croaker macrophages, the expression levels of IRAK4 in both protein and mRNA were significantly inhibited (Fig. 7B). Then we silenced *IRAK4* and examined the production of inflammatory cytokines in LPS-treated miuy croaker macrophages. As shown in Fig. 7B–D, knockdown of IRAK4 significantly inhibited the expression of IL-8, IL-1β, and TNF-α, which produced an effect similar to miR-203 overexpression. These results indicated that miR-203 regulates inflammatory response through suppression of endogenous IRAK4 in miuy croaker. In addition, we have also addressed the generality of
the findings that miR-203 targets IRAK4 in other model animals, such as large yellow croaker, and the results shown in Fig. 7E indicated that miR-203 could also target IRAK4 in large yellow croaker, which indicated the generality of our findings. Taken together, these data convincingly demonstrate that miR-203 inhibits the production of inflammatory cytokines by negative regulation of the miR-203 target gene IRAK4 and modulation of NF-κB signaling, thereby inhibiting the excessive inflammatory responses (Fig. 7F).

**Discussion**

Eradication of bacterial invasion requires timely and appropriate innate immune responses, but excessive induction of inflammatory cytokines production may lead to acute or chronic inflammatory disorders. Hence, various layers of negative mechanisms and regulators play important roles in controlling homeostasis of the immune system and avoiding excessive inflammation. Since microRNA-146a was first reported to be associated with inflammatory responses (19), a series of miRNAs has been documented in the regulation of host immune responses in mammals, whereas it is rare to know the underlying mechanisms of miRNAs in fish species. Miuy croaker, as a member of the Sciaenidae family, is an economically important fish. The study of this species has been conducted in depth from the transcriptome (26) and whole genome (27) to immune genes (28, 29), which makes the miuy croaker an excellent model for studying the immune response of fish. The underlying mechanisms of some molecules in the regulation of immune response have been studied in this species. For instance, IRF9 as a protein regulator has been indicated as a negative regulator involved in the TRIF-mediated NF-κB pathway in miuy croaker (30). Non-coding RNAs, miRNA-3570 (31) and miRNA-214 (23), have been demonstrated to target MyD88 and negatively regulate the inflammation upon pathogen infection in miuy croaker. Nonetheless, the underlying mechanism of the regulatory molecules in TLR-mediated immune response remains to be better understood. Herein, we found that a small non-coding RNA, microRNA-203, is involved in the regulation of IRAK4–NF-κB signaling in miuy croaker, which may enrich the regulation network of immune responses in fish. Importantly, we found that *V. anguillarum* infection could markedly up-regulate miR-203 expression in miuy croaker. Overexpression of miR-203 showed the negative effect on the production
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Figure 6. Overexpression of miR-203 inhibits IRAK-mediated NF-κB signaling upon LPS stimulation. A, HeLa cells were cotransfected with Ctrl, miR-203, Ctrl-i, or miR-203-i, together with pRL-TK Renilla luciferase plasmid, IRAK4 expression plasmid, and luciferase reporter gene NF-κB, IL-8, or IL-1β for 24 h and stimulated with LPS (3 μg/ml) for another 6 h, and then the luciferase activity was measured. For each transfections, the total amount of oligonucleotides was controlled and normalized (final concentration, 100 nM). B, HeLa cells were transfected with pre-miR-203 in a concentration gradient manner, together with luciferase reporter gene NF-κB, IL-8, or IL-1β for 24 h and stimulated with LPS (3 μg/ml) for another 6 h. Afterward, the luciferase activity was measured, and pcDNA6.2 plasmid was used to control the same amount of molecules for transfections. C, the time gradient experiment of the pre-miR-203 plasmid was conducted in HeLa cells treated with or without LPS. Luciferase activity was normalized to Renilla luciferase activity. All data are representative of at least three independent experiments. **, *p < 0.01; *, p < 0.05. Error bars, S.E.

of inflammatory cytokines through IRAK4–NF-κB–mediated signaling by targeting IRAK4, thereby inhibiting excessive immune response. These findings indicate that miR-203 is a new negative regulator of IRAK4–NF-κB–mediated immune response, which suggests a novel miRNA-mediated mechanism for avoiding excessive inflammation in teleost fish.

TLRs, as an important family of pattern recognition receptors, are responsible for the recognition of pathogen infection. This recognition process could induce the production of large amounts of inflammatory cytokines, most notably through the activation of NF-κB (2, 3, 8). Recently, the emerging roles of miRNAs have largely expanded our understanding of the regulation of TLR-mediated immune response. In mammals, miRNAs have been implicated in the TLR-signalizing pathways at several layers. For example, miRNAs, including let-7i, let-7e, miR-105, and miR-19a/b, could directly target TLR expression (17). There are also a lot of miRNAs, through targeting TLR-associated signaling proteins, to regulate TLR signaling response, such as miR-155 targeting MyD88, miR-21 targeting IRAK1, and miR-145 targeting TIRAP (32).

NF-κB is a transcription factor that regulates the expression of many genes, mostly related to immune and inflammatory responses, as well as those genes involved in developmental processes, cell growth, and apoptosis (32). Because of the importance of NF-κB to various biological processes, the regulation of NF-κB activity and activation should be tightly controlled in host cells. In mammals, evidence is growing rapidly that miRNAs play important and complex roles in the regulation of NF-κB signaling. miR-155 has been indicated to negatively regulate the activation of NF-κB in the epithelial cell line (34). miR-26b has also been documented to modulate the NF-κB pathway in alveolar macrophages by regulating PTEN (35). However, little is known about miRNA in the regulation of the NF-κB pathway in fish species. This study demonstrated that miR-203 up-regulated NF-κB signaling by suppressing IRAK4 in LPS-stimulated macrophages, thereby inhibiting the production of inflammatory cytokine and preventing excessive inflammatory responses.

IRAK4, a member of the IL-1 receptor-associated kinase (IRAK) family, is a protein kinase involved in innate immune
responses from Toll-like receptors. Upon TLR activation, IRAK4 could be recruited to MyD88, forming a helical assembly of MyD88-IRAK4-IRAK2/1 complex, which could further activate NF-κB and lead to the transcription of inflammatory genes (8). Hence, IRAK4 is the indispensable pivotal adaptor kinase involved in almost all TLR signaling. Several studies generated IRAK4-inactive kinase knock-in mice and demonstrated the role of kinase activity of IRAK4 in TLR-mediated signaling pathways (36, 37). Evidence has also been documented that inactivation of IRAK4 leads to resistance to LPS-induced septic shock and diminished production of cytokines and chemokines (37). On the basis of these reports, regulation of adaptor kinases might be a highly effective way to maintain host immune response. In mammals, IRAK4 is known to be regulated by miR-132/-212 and miR-146a (20). However, there have not been any related studies reported in fish. Herein, we first reported that miR-203 negatively regulates IRAK4 expression availability at both the mRNA and protein levels, which could enrich the intricate regulation networks for the TLR-associated signaling pathway in fish.

Previous studies reported the involvement of miR-203 in tumorigenesis and the development of bladder, breast, and prostate cancers as well as hepatocellular carcinoma (38). A recent study (39) also described the tumor-suppressive effects of miR-203 in breast cancer, showing that loss of miR-203 led to increased invasion and metastatic potential of the cell system. In mammals, studies have indicated that miR-203 could target MyD88 and may be an important regulator against LPS or mycobacteria infection (40). As reported, a single miRNA could target multiple mRNAs; therefore, in the current study, we demonstrated that miR-203 could target other gene, the pivotal adaptor kinase IRAK4. We found that miR-203 associates with the anti-inflammatory responses and acts as a negative regulator in constraining the production of inflammatory cytokines. The underlying regulation mechanism of miR-203 is through targeting the pivotal adaptor kinase IRAK4 and subsequently negatively regulating NF-κB signaling. Our data provide insight into the role of miR-203 in the innate immune reaction against bacterial infection and may indicate a novel therapeutic approach to combat bacterial infection in fish.
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Materials and methods

Samples and challenge

Healthy miiuy croakers (~750 g) were obtained from Zhoushan Fisheries Research Institute (Zhejiang, China) and raised as reported previously (41). For the stimulation experiment, fish in the experimental group were stimulated with 1 ml of V. anguillarum (1.5 x 10⁸ cfu/ml) or 1 ml of a suspension of ultrapure LPS (1 mg/ml; Sigma) i.p. For comparison, individuals in control groups were kept in separate tanks and correspondingly challenged with 1 ml of physiological water. Fishes were sacrificed at various times, and three individual tissues were collected at each time. All animal experiments were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Research Ethics Committee of the College of Marine Science, Zhejiang Ocean University (approval EC2015011).

Cell culture and transfection

Miiuy croaker macrophages were aseptically isolated from the head kidney of miiuy croaker as reported previously (42) and cultured in L-15 medium (Hyclone) supplemented with 20% fetal bovine serum (Gibco) at 26 °C in 4% CO₂. HEK293 and HeLa cells were cultured in DMEM high-glucose medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) at 37 °C in 5% CO₂. Before transient transfection, cells were seeded into each well of 24-well plates and incubated overnight. After that, cells were transfected with plasmids using Lipofectamine 2000™ (Invitrogen) according to the manufacturer’s protocols. For each transfection experiment, pEGFP plasmid was transfected as the positive control to verify transfection efficiency.

Prediction of miR-203 targeting sites

Software algorithms, including Targetscan (43), miRanda (44), and miRInspektor (45), were used to predict the targets for miR-203. Predictions were ranked based on the predicted efficacy of targeting as calculated using the context and scores of the sites.

Plasmid construction

To construct the IRAK4 3’-UTR reporter vector, the full-length 3’-UTR region of the miiuy croaker IRAK4 gene was amplified using PCR by gene-specific primers with restriction endonuclease sites SacI and XbaI (Table S2) and then inserted into pmirGLO luciferase reporter vector (Promega). Similarly, the 3’-UTR region of the large yellow croaker (Larimichthys crocea) IRAK4 gene was amplified by gene-specific primers with restriction endonuclease sites SacI and XbaI. Then they were inserted into pmirGLO luciferase reporter vector. The mutant of IRAK4 3’-UTR reporter vector was constructed by using Mut Express II Fast Mutagenesis Kit V2 (Vazyme) with specific primers (Table S2). Moreover, both the wildtype and mutant of IRAK4 3’-UTR were inserted into the pEGFP-C1 (Invitrogen), which included the sequence of enhanced green fluorescent protein (EGFP). Additionally, pre-miR-203 plasmid was constructed through PCR amplification and insertion into pcDNA6.2-GW/EmGFP vector (Invitrogen). To construct the IRAK4 expression vector, the full-length CDS region and 3’-UTR of the miiuy croaker IRAK4 gene were amplified by specific primer pairs with the FLAG tag and restriction endonuclease sites BamHI and XbaI and then cloned into pcDNA3.1 vector (Invitrogen). All of the related plasmids were verified by Sanger sequencing and extracted by the Endotoxin-Free Plasmid DNA Miniprep Kit (Tiangen).

miRNA mimics and inhibitors

miR-203 mimics (dsRNA oligonucleotides) and miR-203 inhibitors (single-stranded chemically modified oligonucleotides) and control oligonucleotides were obtained from GenePharma (Shanghai, China). Their sequences are as follows: miR-203 mimics, 5’-GUGAAAUUGUUAGCCACCUUG-3’ (sense) and 5’-AGUGGUCUUACAUUUACUU-3’ (antisense); control mimics, 5’-UUCUGCCAGUGUCAGGTTT-3’ (sense) and 5’-ACUGUACACGCUUGGAGATTT-3’ (antisense); miR-203 inhibitors, 5’-CAAGAGUUCUUACAUUUACAC-3’ (chemically modified by 2’-Ome) and control inhibitors, 5’-CAGUACUUUUGUAGUACAAA-3’. Miiuy croaker macrophages were transfected with a 30–100 nM concentration of each oligonucleotide for 24 h before LPS stimulation using Lipofectamine 2000™.

RNA interference

The si-IRAK4 sequences were 5’-GCAUCAUGUGAGGAGGUUUTT-3’ (sense) and 5’-AAACCUCUCUCAUGUGCTT-3’ (antisense). The scrambled control RNA (si-Ctrl) sequences were 5’-UUCUCGAUGUGACGAGGTTT-3’ (sense) and 5’-ACUGUACACGCUUGGAGGATT-3’ (antisense). Miiuy croaker macrophages were transfected with a 50 nM concentration of each siRNA for up to 48 h before LPS stimulation using Lipofectamine 2000™.

RNA extraction and real-time qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s protocols, and the cDNA was synthesized using the FastQuant RT kit (Tiangen), which included DNase treatment of RNA to eliminate genomic contamination. Primers of the genes were designed, and β-actin was used as an internal control (Table S2). The expression patterns of each gene were performed on a 7500 system (Applied Biosystems) using SYBR® Premix Ex Taq™ (Takara) as described previously (23). For miRNA analysis, the small RNA was extracted by using the mirCute miRNA isolation kit (Tiangen), and the mirCute miRNA FirstStrand cDNA synthesis kit (Tiangen) was applied to reverse transcription of miRNAs. The expression of miR-203 was processed on a 7500 system using the mirCute miRNA qPCR detection kit (Tiangen). The cycling conditions for real-time PCR involved 40 cycles beginning with 15 min at 95 °C followed by 5 s at 95 °C and 30 s at 60 °C. The relative miRNA expression levels were normalized to the 5.8S rRNA internal control, and its primer sequences are listed in Table S2.

Prokaryotic expression and polyclonal antiserum

For prokaryotic expression, the full-length CDS region of miiuy croaker IRAK4 was cloned into a pGEX-4T-1 vector (GE...
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Healthcare) with EcoRI/Xhol sites to construct pGEX-4T-1-IRAK4 plasmid. Then the plasmid pGEX-4T-1-IRAK4 was transformed into the BL21 (DE3) *Escherichia coli* strain and expressed as a protein containing IRAK4 fused with GST. The fusion protein was induced by isopropyl β-D-thiogalactoside and purified by GST-Bind resin chromatography. The purified fusion protein was applied to immunize New Zealand White rabbits (23) to raise a polyclonal anti-IRAK4 antiserum.

**Dual-Luciferase reporter assays**

For miRNA target identification, HEK293 cells were transfected with *IRAK4* 3’-UTR reporter vector, together with miR-203 mimics, inhibitors, controls, or pre-miR-203 plasmid. Reporter luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega). To determine the functional regulation of IRAK4 during LPS stimulation, HeLa cells were transfected with *IRAK4* expression plasmid, together with *Renilla* luciferase plasmid and NF-κB, IL-8, and IL-1β luciferase reporter plasmid, and then stimulated with ultrapure LPS, which was purified by ion-exchange chromatography (2 µg/ml). After treatment with LPS for 12 h, the cells were collected and assayed for reporter activity using the Dual-Luciferase reporter assay system. All of the luciferase activity values were achieved against the *Renilla* luciferase control. For each experiment, three independent experiments were conducted, and each experiment was done in triplicate.

**Western blotting**

To prepare total protein, 24- or 48-h post-transfection cells were collected and lysed and then subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membrane as reported (23). Polyvinylidene difluoride membranes were incubated at 4 °C overnight with anti-FLAG mouse monoclonal antibody, GAPDH monoclonal antibodies (Sigma), or polyclonal anti-IRAK4 antiserum. The following day, the membranes were incubated with the secondary antibody. The immunoreactive proteins were detected by using Western-Bright™ ECL (Advansta), and digital imaging was performed with a cold CCD camera. All of the results are from separate blots to avoid possible problems related to incomplete stripping.

**Statistical analysis**

All of the experiments were performed at least three times independently, with three technical replicates for each experiment. The relative gene expression data were acquired using the 2−ΔΔCT method, and comparisons between groups were analyzed by one-way analysis of variance followed by Duncan’s multiple-comparison tests (33). Results are expressed as mean ± S.E., and differences between means with a p value < 0.05 were considered to be statistically significant.

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