Inducible microRNA-214 contributes to the suppression of NF-κB-mediated inflammatory response via targeting myd88 gene in fish

Received for publication, January 16, 2017, and in revised form, February 16, 2017 Published, JBC Papers in Press, February 24, 2017, DOI 10.1074/jbc.M117.777078

Qing Chu, Yuena Sun, Junxia Cui, and Tianjun Xu

From the Laboratory of Fish Biogenetics & Immune Evolution, College of Marine Science, Zhejiang Ocean University, Zhoushan 316022, China

Edited by Luke O’Neill

Upon recognition of bacterial pathogens by pattern recognition receptors, cells are activated to produce pro-inflammatory cytokines and type I IFN by multiple signaling pathways. Every step of the process must be precisely regulated to prevent dysregulation. MicroRNAs (miRNAs) have been shown to be important regulators with profound effects on inflammatory response. Nevertheless, the miRNA-mediated regulatory mechanism remains unclear in fish species. Here, we addressed the role of miRNA-214 in the bacteria triggered inflammatory response. miR-214 could significantly be up-regulated by Vibrio harveyi and LPS stimulation. Up-regulating miR-214 subsequently inhibits the production of inflammatory cytokines by targeting myd88 to avoid excessive inflammation. Moreover, the negative regulatory mechanism of miR-214 has been demonstrated to be via the myd88-mediated NF-κB pathway. This is the first to focus on miR-214 acting as the negative regulator involved in the bacteria-triggered inflammatory response and thus may provide knowledge on the host-cell regulator responses to microbial infection.

Innate immunity is evolutionarily conserved and acts as the first line of host defense. Recognition of microbial pathogens is an essential process for the initiation of innate immune responses and is mediated by numerous germline-encoded pattern-recognition receptors (PRRs) (1, 2). TLR-like receptors (TLRs), one of the best-characterized PRRs, initiate a wide spectrum of responses from phagocytosis to production of cytokines, which further shape and enhance the inflammatory and adaptive immune responses (3). TLRs trigger the activation of intracellular signaling through a cytoplasmic myeloid differentiation primary response protein 88 (MyD88)-dependent pathway or a MyD88-independent pathway. All mammalian TLRs, with the exception of TLR3, depend at least in part on MyD88 adaptor to transmit signals. In the MyD88-dependent signaling pathway, MyD88 recruits the interleukin-1 receptor-associated kinases (IRAKs) complex, which includes four sub-units: two active kinases (IRAK1 and IRAK4) and two non-catalytic subunits (IRAK2 and IRAKM) (4). Once IRAK1 is phosphorylated, IRAKs associate with TNF receptor-associated factor 6 (TRAF6), leading to activation of NF-κB and MAPKs signaling (5–7). The activation of NF-κB and MAPKs then induces the transcription of various inflammatory genes, including IL-1β, IL-6, and TNFα, leading to inflammatory and innate immune responses (7).

Given that TLRs play vital roles in detecting pathogens and initiating inflammatory responses, strict regulation of the TLR signaling pathways is, therefore, important. Any dysregulation of TLR signaling will disrupt immune homeostasis, which may further induce autoimmune and inflammatory diseases (8). Many molecules have been demonstrated as positive or negative regulators of TLR signaling, including phosphatases, protein kinases, ubiquitin-related proteins, membrane molecules, endosome/lysosome-localized molecules, gene transcription coactivators, antigen-presenting molecules, and even HSP70, HSP70L1, and NGF (9). Nevertheless, the full anti-inflammatory response mechanisms and the processes by which inflammation are resolved remain incompletely understood.

Recently, miRNAs (miRNAs) have emerged as important controllers involved in innate immune response. miRNAs are a class of endogenous, single-stranded, conserved, noncoding small RNAs consisting of ~22 nucleotides. The small RNAs are encoded in genomic clusters and produced by an elaborate expression and processing mechanism (10, 11). The mature miRNA uses its “seed sequence” to bind partially complementary sequences in the 3′-untranslated region (UTR) of target mRNA transcripts, leading to mRNA degradation or translational repression (11). Through regulating gene expression, miRNAs are implicated in regulating diverse biological processes, including embryogenesis, differentiation, tumorigenesis, inflammation, and immunity (12). Presently, more than 1,000 miRNAs have been identified in the human genome, and as much as 60% of all mRNAs have been predicted to be regulated by miRNAs to some extent (13). It is not surprising that miRNAs have been implicated in regulating the components of TLR signaling pathways. For instance, miR-233 and miR-146a have been shown to negatively regulate TLR expression (14), and miR-155 has also been reported to target MyD88 and TBK2 (15, 16).
The Gram-negative bacterium, *Vibrio harveyi*, is a typical Gram-negative pathogen for a wide range of marine animals, and can lead to a variety of vibriosis, a common aquatic animal disease associated with high mortality throughout the world (17). Recently, the development of the miuuy croaker (*Miichthys miuuy*) aquaculture has been hindered by *V. harveyi*, leading to high mortality. Bacterial infection can cause a severe inflammatory response and significant pathology. The role of miRNAs in bacteria-triggered inflammatory response has not been investigated in detail, especially in fish species. Here, we found that the expression of miR-214 is significantly up-regulated following stimulation with *V. harveyi* and LPS in miuuy croaker. Overexpression of miR-214 down-regulates TNF-α, IL-6, IL-1β, and IL-10 expression levels in LPS-exposed macrophages. Further investigation revealed that miR-214 suppresses myd88 expression to regulate the NF-κB signaling pathway and inflammatory cytokines, thereby avoiding excessive inflammation. These studies indicate that miR-214 is up-regulated upon *V. harveyi* infection and acts as a negative regulator of NF-κB signaling by targeting myd88.

**Results**

*V. harveyi* and LPS stimulation induce miR-214 expression

To examine miRNAs that are potentially involved in the regulation of *V. harveyi* infection, a small RNA deep-sequencing analysis of miuuy croaker spleen challenged with *V. harveyi* was performed. From the deep-sequencing data, we selected several miRNAs that were differentially expressed upon stimulation. Among those miRNAs, miR-214 was significantly up-regulated following *V. harveyi* stimulation (data not shown). To further investigate expression profiles of miR-214 upon stimulation, the expression of miR-214 in *V. harveyi*-infected miuuy croaker liver was determined at different times post-infection (Fig. 1A).

LPS, the endotoxin of Gram-negative bacteria, contributes greatly to the structural integrity of the bacteria and elicits strong immune responses in animals. We thus wanted to explore whether miR-214 expression was regulated by LPS stimulation, we used LPS to stimulate miuuy croaker. As shown in Fig. 1B, miR-214 expression was up-regulated quickly from 12 to 48 h and peaked at 36 h after LPS stimulation. Taken together, the up-regulation of miR-214 upon stimulation of *V. harveyi* and LPS suggested that miR-214 may function as a regulator involved in the regulation of the immune response.

**miR-214 suppresses inflammatory cytokines production**

To further determine whether miR-214 expression upon bacteria stimulation could affect the immune response in fish, we have explored the role of miR-214 in inflammatory cytokine production through transfection with miR-214 mimics and miR-214 inhibitor into miuuy croaker macrophages. miRNAs mimics could stimulate naturally occurring mature miRNAs, whereas miRNAs inhibitors blocks the activity of endogenous miRNAs by complementarity. As shown in Fig. 2A, transfection of miR-214 mimics increased miR-214 expression ~800-fold in macrophages, whereas miR-214 inhibitor decreased its expression over 55%. Then, we assessed the contribution of miR-214 mimics and miR-214 inhibitor to the regulation of inflammatory cytokine gene expressions after LPS stimulation. To this end, macrophages were transfected with miR-214, control miRNAs, miR-214 inhibitor, and control inhibitor, respectively, for at least 24 h before LPS stimulation. As shown in Fig. 2B, compared with control miRNA, overexpression of miR-214 significantly reduced the mRNA levels of TNF-α, IL-6, IL-1β, and IL-10 in LPS-exposed macrophages. By contrast, the miR-214 inhibitor caused the enhancement of TNF-α, IL-6, IL-1β, and IL-10 expression (Fig. 2C). These results demonstrated that miR-214 suppresses the production of inflammatory cytokines, including TNF-α, IL-6, IL-1β, and IL-10 in macrophages after LPS stimulation, suggesting miR-214 may play a negative role in response to LPS challenge.

**miR-214 regulates the components of the myd88-dependent signaling cascade**

Upon recognition of infectious pathogens, MyD88 is recruited to all TLRs (except for TLR3) and associates with IRAKs and TRAF6 to transducer signals (20, 21). In mammals, TLR4 recognizes LPS and mediates the MyD88-dependent pathway (22). Although TLR4 in fish is lost or fails to recognize LPS (23), other TLRs may substitute for this function because TLRs in fish display a high variety and distinct features. Compelling evidence shows that signal transduction of the TLR pathway is highly conserved from invertebrates to mammals (24, 25). Therefore, we evaluated proteins in the myd88-dependent signaling cascade to identify the potential target of miR-214. We thus transfected miR-214, control miRNAs, miR-214 inhibitor, and control inhibitor, respectively, into macrophages for up to 24 h, and then monitored mRNA levels of myd88, IRAK1, IRAK4, and TRAF6 by quantitative RT-PCR. As shown in Fig. 3, the expressions of myd88, IRAK1, IRAK4, and TRAF6 were decreased by miR-214 overexpression, whereas increased by miR-214 inhibitor treatment in comparison with the control group. Thus, these results demonstrated that miR-214 suppresses the expression of genes involved in the myd88-dependent pathway cascade.

**myd88 is a target of miR-214**

To further characterize the potential target of miR-214, we used miRNA target prediction program TargetScan (26, 27) to search for potential miR-214 targets, identifying a putative miR-214 binding site in the 3’UTR of myd88 (Fig. 4A). To certify the prediction, we constructed reporter plasmids by cloning miuuy croaker *myd88* 3’UTR into the pmirGLO luciferase
MicroRNA-214 suppresses NF-κB signaling

Figure 2. miR-214 suppresses the mRNA expression of inflammatory cytokines in LPS-stimulated macrophages. A, the macrophages were transfected with control miRNA (miR-Ctrl) or miR-214 (left) and control inhibitor (Ctrl-inhibitor) or miR-214 inhibitor (right) within a final concentration of 100 nM. At 48 h post-transfection, miR-214 expression was measured by qPCR and normalized to 5.8S rRNA. B, the macrophages were transfected with miR-214 mimics or miR-Ctrl. After 24 h, the cells were stimulated with LPS and the expression levels of TNF-α, IL-6, IL-1β, and IL-10 were analyzed by qPCR after another 24 h. C, the macrophages were transfected with miR-214 inhibitor or Ctrl-inhibitor. After 24 h, the cells were stimulated with LPS and the expression levels of TNF-α, IL-6, IL-1β, and IL-10 were analyzed by qPCR after another 24 h. Results are standardized to 1 in control cells. Data are presented as the mean ± S.E. from three independent triplicate experiments. **, p < 0.01; *, p < 0.05 versus the controls.

Figure 3. miR-214 regulates the expression of myd88, IRAK1, IRAK4, and TRAF6. The macrophages were transfected with miR-214 or miR-Ctrl and miR-214 inhibitor or Ctrl-inhibitor (final concentration, 100 nM). After 48 h, myd88 (A), IRAK1 (B), IRAK4 (C), and TRAF6 (D) mRNA levels were determined by qPCR and RT-PCR (E). Results are standardized to 1 in control cells, and mRNA levels are normalized to β-actin. Data are presented as the mean ± S.E. from three independent triplicate experiments. *, p < 0.05 versus the controls.

reporter vector within the mutation at the miR-214 binding site as control, and then transfected the plasmids into HEK293 cells for Dual-Luciferase reporter assays. As shown in Fig. 4B, the relative luciferase activity was reduced by 62% following cotransfection with miR-214 mimics compared with transfection with control miRNAs (miR-Ctrl). By contrast, no change of luciferase was observed in cells transfected with mutant-type constructs. This result was subsequently confirmed by concentration and time gradient experiments (Fig. 4D). Moreover, as shown in Fig. 3C, miR-214 mimics and inhibitor were used to further verify the down-regulation mechanism, and results revealed that inhibition of luciferase activity was attenuated after cotransfection with the miR-214 inhibitor.
In addition, pre-miR-214 was predicted, and its sequence was cloned into the pcDNA6.2-GW/EmGFP vector to construct the pre-miR-214 plasmid (Fig. 4A). Subsequently, we sought to transfect the pre-miR-214 plasmid, together with wild-type or mutant-type myd88-3’UTR into HEK293 cells to demonstrate the down-regulation mechanism. After 48 h, luciferase activity was determined and normalized to Renilla luciferase activity. C, HEK293 cells were cotransfected with myd88-3’UTR (WT), together with miR-214 or miR-Control and miR-214 inhibitor or Ctrl-inhibitor. Each assay was transfected with equal amounts of oligonucleotides (final concentration, 100 nM). After 24 h, luciferase activity was determined and normalized to Renilla luciferase activity. D, HEK293 cells were cotransfected with myd88-3’UTR (WT), together with miR-214 and miR-Control in concentration (left) and time (right) gradient manners. E, HEK293 cells were cotransfected with myd88-3’UTR (WT), together with pre-miR-214 plasmid and control plasmid in concentration (left) and time (right) gradient manners. The luciferase activity value was achieved against the Renilla luciferase activity. F, HEK293 cells were cotransfected with pIZ/EGFP-myd88-3’UTR or empty vector, together with miR-214 or miR-Control and pre-miR-214 or pcDNA6.2 plasmid. At 48 h post-transfection, the fluorescence intensity was evaluated. Data are presented as the mean ± S.E. from three independent triplicate experiments. **, p < 0.01 versus the controls.

In addition, pre-miR-214 was predicted, and its sequence was cloned into the pcDNA6.2-GW/EmGFP vector to construct the pre-miR-214 plasmid (Fig. 4A). Subsequently, we sought to transfect the pre-miR-214 plasmid, together with wild-type or mutant-type myd88-3’UTR into HEK293 cells to demonstrate the down-regulation mechanism. After 48 h, the Dual-Luciferase assays were conducted to measure the relative luciferase activity, which was reduced by 43.6% compared with the control (Fig. 4B). This result was confirmed by concentration and time gradient experiments (Fig. 4C). Furthermore, both miR-214 mimics and pre-miR-214 plasmid down-regulated GFP gene expression when the myd88-3’UTR was cloned into the pIz/EGFP vector in HEK293 cells, whereas no change of GFP gene expression was observed within the mutant-type constructs (Fig. 4D). The above data suggested that miR-214 targets the 3’UTR of myd88, and myd88 is a new target of miR-214.

**miR-214 decreases the abundance of myd88 at both the mRNA and protein levels**

Given that miRNAs down-regulate the target genes through mRNA degradation or inhibition of translation, we next investigated which mechanism results in the suppression of myd88 by miR-214 in fish. To this end, we transfected using miR-214 mimics or control miRNA into miiuy croaker macrophages, and then measured the mRNA and protein levels of myd88. As shown in Fig. 5A, transfection with miR-214 mimics markedly decreased MyD88 abundance at both the mRNA and protein levels in a dose-dependent manner. As a comparison, transfection with the miR-214 inhibitor enhanced myd88 abundance at both the mRNA and protein levels as compared with that in cells transfected with the control inhibitor (Fig. 5B). These results indicated that miR-214 can decrease the abundance of endogenous myd88. Additionally, the myd88 expression plasmid was constructed within the full-length CDS region and 3’UTR of the miiuy croaker myd88 gene. Given that the miRNA processing system is conserved from invertebrates to vertebrates (28, 29), we then sought to transfect with the myd88 expression plasmid, together with the pre-miR-214 plasmid into HEK293 cells. To construct the myd88 expression plasmid, the full-length CDS region and 3’UTR of the miiuy croaker myd88 gene was amplified by specific primer pairs and cloned into the pcDNA6.2-GW/EmGFP vector to construct the pre-miR-214 plasmid (Fig. 4A).
MicroRNA-214 suppresses NF-κB signaling

Figure 5. miR-214 treatment decreases the abundance of myd88 at both mRNA and protein level. A, the macrophages were transfected with miR-214 or miR-Ctrl within the concentration gradient. After 48 h, myd88 mRNA levels were determined by qPCR and normalized to β-actin (left), and myd88 protein levels were determined by Western blot and normalized to β-actin (right). B, the macrophages were transfected with miR-214 inhibitor or Ctrl-inhibitor within concentration gradient. After 48 h, myd88 mRNA levels were determined by qPCR and normalized to β-actin (left), and myd88 protein levels were determined by Western blot and normalized to β-actin (right). C, HEK293 cells were cotransfected with myd88 expression plasmid, together with pre-miR-214 and control plasmid. After 48 h, myd88 mRNA levels were determined by qPCR and normalized to β-actin (left), and myd88 protein levels were determined by Western blot and normalized to β-actin (right). Data are presented as the mean ± S.E. from three independent triplicate experiments. **, p < 0.01; *, p < 0.05 versus the controls.

miR-214 suppresses LPS-induced inflammation through myd88

To explore the role of myd88 involved in LPS-induced inflammatory response, we silenced myd88 and then examined the production of inflammatory cytokines in macrophages treated with LPS stimulation. siRNA effectively inhibited the expression levels of myd88 protein and mRNA (Fig. 6, A and B). As shown in Fig. 6C, knockdown of myd88 significantly decreased the levels of TNF-α mRNA in macrophages upon LPS stimulation, which produced an effect similar to that of miR-214 overexpression. Similar results were observed among the expression levels of IL-6, IL-1β, and IL-10 (Fig. 6, D–F). These results indicated that miR-214 regulates inflammatory response through suppression of endogenous myd88, thereby inhibiting inflammatory cytokine production.

miR-214 suppresses myd88-mediated NF-κB pathway

Previous studies demonstrated that compared with other TLR adaptors, the structure of MyD88 is well conserved and its homologs in fish species may function similarly to mammalian counterparts (9, 10). In zebrafish, myd88 has been reported to be involved in the positive regulation of the NF-κB promoter, and similar reports were also demonstrated in salmonids (11, 12). We next investigate the regulatory role of myd88 in miuy croaker. To this end, the myd88 expression plasmid containing the full-length CDS region and 3'UTR of the miuy croaker myd88 gene was constructed and transfected into HEK293 cells to measure its regulatory function. As shown in Fig. 7A, overexpression of myd88 efficiently up-regulated the NF-κB reporter gene. Given that miR-214 targets myd88 and down-regulates its expression, we then tested whether miR-214 is a negative regulator in the NF-κB pathway. We thus transfected using the myd88 expression plasmid, together with miR-214 mimics and miR-214 inhibitor, into HEK293 cells and each assay was transfected with an equal amount of oligonucleotides. As expected, we found that overexpressed miR-214 could significantly down-regulate the NF-κB reporter gene, whereas the down-regulation mechanism was attenuated after cotransfection with miR-214 inhibitor (Fig. 7B). To confirm the above results, the concentration and time gradient experiments were conducted (Fig. 7, C and D). These results indicated that miR-214 suppresses the NF-κB pathway by down-regulating myd88. Additionally, a signaling of miR-214 in modulating myd88 expression to down-regulate the NF-κB pathway has also been performed (Fig. 7E).

Discussion

miRNAs are involved in the regulation of multiple biological processes, whereas the role of miRNAs in the inflammatory response of fish to bacterial challenge has not been investigated. In this study, we addressed the role of miRNA-regulated pathways in a bacteria triggered inflammatory response. We found that miR-214 could be up-regulated in response to V. harveyi infection, as well as LPS stimulation in fish. Up-regulation of miR-214 suppressed the NF-κB pathway by targeting myd88, therefore inhibiting inflammatory cytokines production, including IL-1β, IL-6, TNF-α, and IL-10. These findings not only indicate that miR-214 is a new negative regulator of MyD88-mediated immune response but also suggest a novel mechanism for avoiding excessive inflammation in teleost fish. TLRs are the best-characterized PRRs that detect conserved microbial components during infection and then initiate inflammatory responses. After recognition of the pathogen-associated molecular pattern, TLRs initiate innate immune responses by the MyD88-dependent signaling pathway and MyD88-independent signaling pathway, which then induce
pro-inflammatory cytokine and type I interferon (IFN) production to control inflammatory and immune responses (3, 4). However, excessive activation of TLR signaling could disrupt immune homeostasis, thereby inducing some diseases, such as autoimmune diseases, chronic inflammatory diseases, or cancer (8). Therefore, it is especially important to use precise regulation of the TLR signaling pathways. Negative regulators of TLR signaling could intersect at almost every step of the TLR signaling pathway. For instance, NLRX1 was shown to function as intracellular PRRs to suppress TLR signaling through interaction with TRAF6 and IKK complexes (31). With the exception of TLR3, all mammalian TLRs utilize MyD88 to commence

Figure 6. The expression levels of myd88 and inflammatory cytokines after myd88 interference. Miiuy croker macrophages were transfected with control siRNA (si-Ctrl) or siRNA against myd88 (si-MyD88). A, after 48 or 72 h, myd88 protein levels were determined by Western blot and normalized to β-actin. B, after 48 h, myd88 mRNA levels were determined by qPCR and normalized to β-actin. C and D, after 24 h transfection with si-Ctrl or si-MyD88, macrophages were then stimulated with LPS for another 6 or 12 h and the expression of TNF-α (C), IL-6 (D), IL-1β (E), and IL-10 (F) were determined. Data are presented as the mean ± S.E. from three independent triplicate experiments. **, p < 0.01; *, p < 0.05 versus the controls.

Figure 7. Overexpression of miR-214 suppresses myd88-mediated NF-κB pathway. A, HEK293 cells were cotransfected with myd88 expression plasmid, pRL-TK Renilla luciferase plasmid, together with NF-κB reporter genes. After 48 h, the luciferase activity was measured and results presented relative to the luciferase activity in control cell. B, miR-214 or miR-Ctrl and miR-214 inhibitor or Ctrl-inhibitor were cotransfected with myd88 expression plasmid, pRL-TK Renilla luciferase plasmid, together with luciferase reporter NF-κB into HEK293 cells. After 24 h, luciferase activity was measured and results presented relative to the luciferase activity in the control cell. C, the concentration gradient experiment of miR-214 (left) or pre-miR-214 plasmid (right) was conducted. After 24 h, the luciferase activity was measured. D, the luciferase activity was measured after 24 or 48 h cotransfection with miR-214 (left) or pre-miR-214 plasmid (right). E, the proposed model for miR-214 of inflammatory cytokine secretion via myd88. Data are presented as the mean ± S.E. from three independent triplicate experiments. **, p < 0.01; *, p < 0.05 versus the controls.
MicroRNA-214 suppresses NF-κB signaling

signaling, activating NF-κB and MAPK to regulate host-cell response to pathogens (4). Thus, it may be the most effective to directly regulate MyD88, the TLR downstream adaptor, in host immune response. Accumulated evidence has suggested that MyD88 was strictly regulated during inflammatory response. For instance, IRF4 interacts with MyD88 as a negative regulator of TLR signaling (32).

Recently, miRNAs have emerged as important controllers of innate immune response (33). The present studies showed that miRNAs play vital roles in the regulation of genes of the immune system, including macrophages, microglia, dendritic cells, and T cells (34), and sets of miRNAs have been demonstrated to participate in the modulation of TLR signaling. miRNAs regulate TLR-signaling pathways at multiple layers, including direct regulation of TLR expression, TLR-associated signaling proteins, and TLR-induced transcription factors and functional cytokines. With regard to direct regulation of TLR expression, several miRNAs have been reported. The let-7 miRNA family, including let-7e and let-7i (35), can regulate TLR4 expression, miR-223 is a regulator for both TLR4 and TLR3 (36), and miR-143 can inhibit the expression of TLR2 (37). For the regulation of TLR-associated signaling proteins, recent reports demonstrated that miR-155 can negatively regulate TLR signaling pathways by targeting key signaling proteins, such as MyD88 and TBK2 (15, 16). Moreover, miR-146 negatively regulates the MyD88-mediated NF-κB pathway post-bacterial infection through targeting IRAR1 and TRAF6 in THP-1 macrophage cells (38). As the important TLR downstream adaptor, MyD88 have also been reported to be regulated by an array of miRNAs, such as miR-146b, miR-155, miR-200b, miR-200c, miR-21, miR-149, and miR-203 (14). Additionally, large amounts of miRNAs are involved in the regulation of transcription factors. For instance, miR-223 targets IKKa and miR-199 is a regulator of IKKB, which all participate in the regulation of NF-κB activity (39, 40). In accordance with these earlier miRNAs, our findings showed that miR-214 could inhibit the expression of miR-214, followed by inhibitory inflammatory cytokine production in miuuy croaker macrophages.

Miiuy croaker, a member of the Sciaenidae family, is an economically important marine fish. Evidence indicates that miuuy croaker has been studied in depth from transcriptome (41, 42), whole-genome (43), to functional genes, which left miiuy croaker as a new model for studying the immune system in fish. V. harveyi, as the marine Gram-negative bacteria, is a serious pathogen for a wide range of marine animals. In response to bacterial infection, innate immunity, and a series of inflammatory response is the main defense system in fish (30). However, dysregulation of inflammatory response may disrupt immune homeostasis, leading to some diseases and even death. In this study, we found that V. harveyi infection could significantly up-regulate the expression of miR-214, which could prevent an excessive inflammatory response through inhibiting the production of inflammatory cytokines. These findings demonstrate miR-214 acts as the negative regulator involved in the bacteria-triggered inflammatory response, which can enrich miRNA-mediated feedback on the regulatory mechanisms of the innate signaling pathway.

Materials and methods

Sample and challenge

Healthy miiuy croakers (~750 g) were obtained from Zhoushan Fisheries Research Institute (Zhejiang, China) and raised in aerated seawater tanks at 25 °C for at least 1 week. For the stimulation experiment, briefly, these healthy fish were randomly divided into two groups in which the experimental individuals were challenged with 1 ml of V. harveyi (1.5 × 10⁸ cfu/ml) or 1 ml of suspension of LPS (1 mg/ml, Sigma) intra-peritoneally and the other individuals kept in separate tanks were corresponding challenged with 1 ml of physiological water as the control. Fish were sacrificed in 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 (number EC2015011).

Macrophage culture

For the macrophage isolation, the head kidneys from juvenile miiuy croaker were collected aseptically. Tissues were minced thoroughly with scissors and pushed carefully through a 100-μm nylon mesh in L-15 medium containing penicillin (100 IU/ml), streptomycin (100 mg/ml), 2% fetal bovine serum (FBS), and heparin (20 units/ml) to give a single cell suspension. The filtered cell suspension was loaded onto 34/51% Percoll (Pharmacia, USA) density gradient, and then centrifuged at 400 × g for 40 min at 4 °C. Subsequently, the supernatant was removed and the cells at the interfaces were obtained with care and washed twice in L-15 medium at 300 × g for 10 min at 4 °C. Macrophages were cultured in L-15 containing 0.1% FBS at 26 °C, 4% CO2. The next day, the cell pellet was re-suspended in fresh complete L-15 medium supplemented with 20% FBS.

RNA isolation and real-time quantitative PCR

Total RNA and small RNAs (<200 nucleotides) were isolated with TRIzol reagent (Invitrogen) and their miRcute miRNA Isolation Kit (Tiangen), respectively, following the manufacturer’s protocol. Real-time quantitative PCR (qPCR) was performed on a 7300 real-time PCR system (Applied Biosystems, USA) as previously described (18). The relative expression level of mRNA was normalized by β-actin expression, whereas miRNA was normalized by 5.8S rRNA. All amplification reactions were carried out within a triplicate well of each sample and sequences of mRNA and miRNA primers listed in supplemental Table S1.

Plasmid construction and transfection

To construct the myd88 expression vector, the full-length CDS region and 3’UTR of the miiuy croaker myd88 gene was amplified by specific primer pairs harboring a FLAG tag and restricted endonuclease sites KpnI and EcoRI, and then inserted into pcDNA3.1 vector (Invitrogen). To construct the myd88-3’UTR reporter vector, the full-length 3’-UTR region of myd88 was amplified from cDNA of miiuy croaker. The PCR product was digested within Nhel and Sall, respectively, which
was then cloned into the pmirGLO luciferase reporter vector (Promega). The mutant-type of myd88-3’UTR reporter vector was conducted using Mut Express II Fast Mutagenesis Kit V2 (Vazyme) with specific primers (supplemental Table S1). Additionally, myd88-3’UTR or mutant-type was cloned into the pIZ/V5-His vector (Invitrogen), which contained the enhanced green fluorescent protein (GFP) sequence. To construct pre-
microRNA vector, the pre-miR-214 sequence were amplified by PCR and then cloned into pcDNA6.2-GW/EmGFP vector (Invitrogen). All recombinant plasmids were extracted through the Endotoxin-free Plasmid DNA Miniprep Kit (Tiangen) and confirmed by Sanger sequencing before the Dual-Luciferase reporter assay. Before transient transfection, HEK293 cells were seeded in 24-well plates for 24 h. Cells were subsequently transfected with 100 ng of plasmids using Lipofectamine 2000™ (Invitrogen), according to the manufacturer’s instructions.

miRNA mimics and inhibitors

miR-214 mimics (dsRNA oligonucleotides), miR-214 inhibitor (single-stranded oligonucleotides chemically modified by 2’-Ome), and control oligonucleotides were commercially synthesized by GenePharma (Shanghai, China). Their sequences are as follows: miR-214 mimics, sense, 5’-ACACGAGCGACAGGGCAG-3’; antisense, 5’-GCCUGUCUGCUGCU(UU)-3’; control mimics, sense, 5’-UCCGAGCGACAGGGCAG-3’; antisense, 5’-GCCUGUCUGCUGCU(UU)-3’. miR-214 inhibitor, 5’-UCUGGCGUCUGCUCUGCUGU[3’]; and inhibitors control, 5’-CAGUACUUUUGUGUAUGUCAA-3’. HEK293 cells or macrophages were seeded into 24-well plates, and incubated overnight. Cells were subsequently transfected with 30–100 nM of each oligonucleotide 24 h before LPS stimulation using Lipofectamine 2000™ (Invitrogen).

RNA interference

The myd88-specific siRNA was 5’-GCUCGAAACAAA-CGCCCCUATT3’ (sense) and 5’-UAAAGCUGUUGUUC-GAGCTT-3’ (antisense). The scrambled control RNA sequences were 5’-UUCGGCAACGUGACGGTT-3’ (sense) and 5’-AGUGACGCGUUGAGAATT-3’ (antisense). The myd88-specific siRNA transfection was performed with Lipofectamine 2000™ (Invitrogen). Macrophages were seeded into 24-well plates, and incubated overnight. Cells were subsequently transfected with 100 nM of each siRNA for 24 h before LPS stimulation.

Prokaryotic expression and polyclonal antiserum

For prokaryotic expression, the full-length CDS region of miyuy croaker myd88 was cloned into EcoRI/XhoI sites of the pGEX-4T-1 vector (GE Healthcare) to construct the pGEX-4T-1-myd88 plasmid. Subsequently, the plasmid pGEX-4T-1-myd88 was transformed into the Escherichia coli BL21(DE3) strain and expressed as a protein containing myd88 fused with GST. The fusion protein was induced by isopropyl 1-thio-β-D-galactopyranoside and purified by GST-Bind Resin chromatography. The purified fusion protein was applied to immunize New Zealand White rabbits to raise a polyclonal anti-myd88 antiserum. Validation for polyclonal antiserum is documented in supplemental Fig. S1.

**MicroRNA-214 suppresses NF-κB signaling**

For miRNA target identification, HEK293 cells were cotransfected with wild-type or mutant type myd88-3’UTR luciferase reporter vector, along with miR-214 mimics, inhibitors, and controls or pre-miR-214 plasmid. Additionally, HEK293 cells were cotransfected with the NF-κB luciferase reporter plasmid, pRL-TK Renilla luciferase plasmid, myd88 expression plasmid, along with miR-214 mimics, inhibitors, and controls or pre-miR-214 plasmids for Dual-Luciferase reporter assay. After 24 or 48 h, the cells were collected and assayed for reporter activity using the Dual-Luciferase Reporter System (Promega) following the manufacturer’s instructions and the relative luciferase activity value was achieved against the Renilla luciferase control. The results shown were done in triplicate for each experiment, and three independent experiments were conducted.

**Western blotting**

After 48 h post-transfection, total HEK293 cellular lysates or macrophages lysates were generated. The soluble protein concentrations were measured with a BCA Protein Assay Kit (Thermo Scientific), and an equal amount of protein was loaded for SDS-PAGE. Proteins were transferred onto PVDF membranes (Pall Corporation) in a semi-dry manner (Bio-Rad Trans Blot Turbo System). Membranes were blocked for 1 h with 5% dried skimmed milk powder in 100 mM TBST. Then, the membranes were incubated at 4 °C overnight with anti-FLAG mouse monoclonal antibody (Beyotime) or polyclonal anti-myd88 antiserum and zebrafish β-actin monoclonal antibodies (Beyotime), respectively. The following day, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase at room temperature for 60 min (Beyotime). The immunoreactive proteins were detected using BeyoECL Plus (Beyotime) and digital imaging was performed with a cold CCD camera. The results shown were done in triplicate for each experiment, and three independent experiments were conducted. All of the results are from separate blots to avoid possible problems related to incomplete stripping.

**Statistical analysis**

All experiments were repeated three times. Data on relative gene expression were obtained using the 2-ΔΔCt method, and comparisons between groups were analyzed by one-way analysis of variance followed by Duncan’s multiple comparison tests (19). All data were presented as the mean ± S.E., significant differences between groups were determined by two-tailed Student’s t test.

**Author contributions**—Q. C. and T. X. conceived and designed the experiments; Q. C., J. C., and T. X. performed the experiments; Q. C. and T. X. analyzed the data; Q. C., Y. S., J. C., and T. X. contributed reagents/materials/analysis tools; and Q. C., Y. S., J. C., and T. X. wrote the paper.
MicroRNA-214 suppresses NF-κB signaling

References

1. Takeuchi, O., and Akira, S. (2010) Pattern recognition receptors and inflammation. *Cell* **140**, 805–820
2. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**, 373–384
3. Kawai, T., and Akira, S. (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–650
4. Takeda, K., Kaisho, T., and Akira, S. (2003) Toll-like receptors. *Annu. Rev. Immunol.* **21**, 335–376
5. Lang, C. H., Silvis, C., Deshpande, N., Nystrom, G., and Frost, R. A. (2003) Endotoxin stimulates *vivo* expression of inflammatory cytokines tumor necrosis factor α, interleukin-1β, -6, and high-mobility-group protein-1 in skeletal muscle. *Shock* **19**, 538–546
6. Koziczak-Holbro, M., Glück, A., Tschopp, C., Mathison, J. C., and Gram, H. (2008) IRAK-4 kinase activity-dependent and -independent regulation of lipopolysaccharide-inducible genes. *Eur. J. Immunol.* **38**, 778–796
7. Rakoff-Nahoum, S., and Medzhitov, R. (2009) Toll-like receptors and cancer. *Nat. Rev. Cancer* **9**, 57–63
8. Kondo, T., Kawai, T., and Akira, S. (2012) Dissecting negative regulation of Toll-like receptor signaling. *Trends Immunol.* **33**, 449–458
9. Li, Y., and Shi, X. (2013) MicroRNAs in the regulation of TLR and RIG-I pathways. *Cell. Mol. Immunol.* **10**, 65–71
10. Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., and Kim, V. N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060
11. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–223
12. Stefani, G., and Slack, F. J. (2008) Small non-coding RNAs in animal development. *Nat. Rev. Mol. Cell Biol.* **9**, 219–230
13. Kozomara, A., and Griffiths-Jones, S. (2011) miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* **39**, D152–157
14. He, X., Jing, Z., and Cheng, G. (2014) MicroRNAs: new regulators of innate immunity: update on Toll-like receptors. *Cell. Mol. Immunol.* **11**, 197–206
15. Tang, B., Xiao, B., Liu, Z., Li, N., Zhuo, Y. Z., and Mao, X. H. (2010) Identification of MyD88 as a novel target of miR-155, involved in negative regulation of *Helicobacter pylori*-induced inflammation. *FEBS Lett.* **584**, 1481–1486
16. Huang, R. S., Hu, G. Q., Lin, B., Lin, Z. Y., and Sun, C. C. (2010) MicroRNA-155 silencing enhances inflammatory response and lipid uptake in oxidized low-density lipoprotein-stimulated human THP-1 macrophages. *J. Invest. Med.* **58**, 961–967
17. Austin, B., and Zhang, X. H. (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett. Appl. Microbiol.* **43**, 119–124
18. Chu, Q., Sun, Y., Bi, D., Cui, J., and Xu, T. (2017) Up-regulated of miR-8159-5p and miR-217-5p by LPS stimulation negatively co-regulate TLR1 in miioy croaker. *Dev. Comp. Immunol.* **67**, 117–125
19. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^-delta CT method. *Methods* **25**, 402–408
20. Akira, S., Yamamoto, M., and Takeda, K. (2003) Role of adapters in Toll-like receptor signalling. *Biochem. Soc. Trans.* **31**, 637–642
21. O’Neill, L. A., and Bowie, A. G. (2007) The family of five: TIR-domain-containing adaptor protein is partially responsible for lipopolysaccharide insensitivity in zebrafish with a highly conserved function of MyD88. *J. Immunol.* **185**, 3391–3400
22. Rebl, A., Rebl, H., Liu, S., Goldammer, T., and Seyfert, H. M. (2011) Salmonid Tollip and MyD88 factors can functionally replace their mammalian orthologues in TLR-mediated trout SAA promoter activation. *Dev. Comp. Immunol.* **35**, 81–87
23. Friedman, R. C., Farh, K. K., Burge, C. B., and Bartel, D. P. (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105
24. Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005) (2004) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15–20
25. Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F., and Hannon, G. J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature* **432**, 231–235
26. Michlewski, G., Guil, S., Semple, C. A., and Cáceres, J. F. (2008) MicroRNA-214 suppresses NF-κB signaling by targeting TRAF6 and IκB. *Immunity* **34**, 843–853
27. Negishi, H., Ohba, Y., Yanai, H., Takaoka, A., Honma, K., Yui, K., Matsuyama, T., Taniguchi, T., and Honda, K. (2005) Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 15989–15994
28. Naik, M. A., Sato, M., and Chen, F. K. (2011) MicroRNA in TLR signaling and endotoxin tolerance. *Cell. Mol. Immunol.* **8**, 388–403
29. Quinn, S. R., and O’Neill, L. A. (2011) A trio of microRNAs that control Toll-like-receptor signalling. *Int. Immunol.* **23**, 421–425
30. O’Hara, S. P., Splinter, P. L., Gajdus, G. B., Trussoni, C. E., Fernandez-Zapico, M. E., Chen, X. M., and LaRussa, N. F. (2010) NFκB p50-CCAAT enhancer-binding protein β (C/EBPβ)-mediated transcriptional repression of microRNA let-71 following microbial infection. *J. Biol. Chem.* **285**, 216–225
31. Heikham, R., and Shankar, R. (2010) Flanking region sequence information to refine microRNA target predictions. *J. Biosci.* **35**, 105–118
32. Guo, H., Chen, Y., Hu, X., Qian, G., Ge, S., and Zhang, J. (2013) The regulation of Toll-like receptor 2 by miR-143 suppresses the invasion and migration of a subset of human colorectal carcinoma cells. *Mol. Cancer* **12**, 77
33. Taganov, K. D., Boldin, M. P., Chang, K. J., and Baltimore, D. (2006) NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 12481–12486
34. Chen, R., Alvero, A. B., Silasi, D. A., Kelly, M. G., Fest, S., Visintin, I., Leiser, A., Schwartz, P. E., Rutherford, T., and Mor, G. (2008) Regulation of IKKβ by miR-199a affects NF-κB activity in ovarian cancer cells. *Oncogene* **27**, 4712–4723
35. Li, T., Morgan, M. J., Choksi, S., Zhang, Y. S., and Liu, Z. G. (2010) MicroRNAs modulate the noncanonical transcription factor NF-κB pathway by regulating expression of the kinase IKKα during macrophage differentiation. *Nat. Immunol.* **11**, 799–805
36. Che, R., Wang, R., and Xu, T. (2014) Comparative genomic of the teleost cathespin B and H and involvement in bacterial induced immunity of miioy croaker. *Fish Shellfish Immunol.* **41**, 163–171
37. Chu, Q., Gao, Y., Xu, G., Wu, C., and Xu, T. (2015) Transcripome comparative analysis revealed poly(I:C) activated RIG-I/MDA5-mediated signaling pathway in miioy croaker. *Fish Shellfish Immunol.* **47**, 168–174
38. Xu, T., Xu, G., Che, R., Wang, R., Wang, Y., Li, J., Wang, S., Shu, C., Sun, Y., Liu, T., Liu, J., Wang, A., Han, I., Chu, Q., and Yang, Q. (2016) The genome of the miioy croaker reveals well-developed innate immune and sensory systems. *Sci. Rep.* **6**, 21902