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Short communication

# IRF9 as a negative regulator involved in TRIF-mediated NF-κB pathway in a teleost fish, *Miichthys miiuy*

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ABSTRACT

Proinflammatory cytokines and type I IFNs were produced by TLR signaling and these responses are crucial for host defensive responses against pathogens. In order to avoid harmful and inappropriate inflammatory responses, there are multiple mechanisms to negatively regulate TLR signaling. In this paper, we have firstly studied IRF9 functions as a negative regulator involved in TRIF-mediated NF-κB pathway. Moreover, we found inhibitory effect of IRF9 primary depends on DBD domain. Interestingly, we also demonstrated that else mutants of IRF9, except for IRF9-ΔDBD, have different inhibitory effects upon TRIF-mediated NF-κB pathway. This study provides a novel evidence about the negatively regulation of innate immune signaling pathway in teleost fish. In addition, this finding provides new insights into the regulatory mechanism in mammals.



## 1. Introduction

The immune defense system has evolved in vertebrates to eliminate infective pathogens when the body is constantly threatened by invasion of microorganisms; this system consists of innate and acquired immunity (Akira et al., 2006). The innate immune response as the first line of body could protect the host from invading microbial pathogens, and its activation may be as a precondition to trigger the acquired immunity (Akira et al., 2001). The innate immune response against invading pathogens depends on pattern-recognition receptors (PRRs) to sensing of pathogen-associated molecular patterns (PAMPs) (Thompson et al., 2011; Kawai and Akira, 2010). These PRRs mainly include TLRs, NLRs, RLRs, and CLRs (Kawai and Akira, 2010; Kingeter and Lin, 2012; Eisenacher and Krug, 2011; Elinav et al., 2011). Then, each PRR recruits downstream adaptor molecule to transmit signals and activates distinct transcription factors, leading to the production of type I interferons (IFNs) and cytokines (Werts et al., 2006; O'Neill and Bowie, 2007). Therefore, the innate immune response is indispensable for controlling microbial infection. However, excessive immune response could lead to many inflammatory and autoimmune diseases, so varieties of regulatory factors are need to tightly regulate the TLR signaling pathway to maintain immune balance.

TLRs, as an important PRR, play a critical role in host defense against pathogenic microbes by recognizing PAMPs (Medzhitov, 2007). Until now, there are at the least 26 members of TLRs with various names identified from different species, containing fish, birds, amphibians, reptiles, and mammals. However, fish TLRs reveal distinct and high variety characteristics; TLR18-20 and TLR23-28 were identified only exist in fish, and certain nonmammalian TLRs have been detected in fish (Wang et al., 2016). Signaling through TLRs can be broadly separated into two pathways that are associated with downstream activation of NF-κB, MAPKs, and IFN regulatory factors (IRFs) (O'Neill and Bowie, 2007). All TLRs have a Toll/IL-1R domain through which interacte with TLR adaptor proteins. To date, five of adaptor proteins have been identified, including MyD88, MAL/TRIAP, TRIF, TRAM, and SARM respectively (O'Neill and Bowie, 2007). And these adaptors promote the activation of the MyD88-dependent and MyD88-independent pathways. Almost all TLRs use MyD88 adaptor to transmit signals and activate the NF-κB and other transcription factors that facilitate expression of classic proinflammatory cytokines (Lin et al., 2010). However, TRIF is only used by both TLR3 and TLR4 to mediate a MyD88-independent pathway which drives the expression of type I IFN and proinflammatory cytokines (Gay et al., 2014; Alexopoulou et al., 2001), thereby leading the body to producing inflammatory response. However, excessive inflammatory response can cause damage on body. In order to maintain immune balance, signal pathway must be tight regulated in the immune response. Recent work has revealed complex regulation of TLR signal transduction at numbers of different levels including phosphorylation, degra-

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dation, and sequestration of signaling molecules (Komuro et al., 2008). For example, MyD88s, have been reported to be an endogenous negative regulator in MyD88-dependent singaling pathway (Burns et al., 2003). In addition, SOCS-1 as a critical regulator in the immune systems and has been shown to negatively regulate the IFN signaling pathways (Nie et al., 2014).

TRIF facilitates TLR3 and TLR4 signaling and activate the transcription factors, NF-κB and IRF3 leading to the production of proinflammatory cytokine and type I IFN. Previous studies have shown many cases of negative regulation of TRIF involved in above signaling pathway. For instance, SARM could negatively regulate the TRIF-mediated signaling pathway (Carty et al., 2006). And ADAM15 is a negative regulator of TLR3 and TLR4 signaling and involved in the mechanism of TRIF degradation (Ahmed et al., 2013). Although some reports have confirmed about the regulation mechanism of TRIF, but there is no report that IRF family could regulate TRIF.

IRF9 belongs to IRF family, and it is first recognized from a component called ISGF3 (Veals et al., 1992). And in the fish genome, alike to the mammals, it has the total IRF family members exhibiting a clear orthologous relationship with mammalian the counterparts (Stein et al., 2007). The previous research demonstrated that, in IRF9-deleted of the mouse cells, the activation of ISGs by IFNα or IFNγ established the host antiviral state are all showed certain impaired, and the corresponding ISRE-binding activities by the IFNs are absent, thus the result indicating that IRF9 is a vital type I IFN and type II IFN response (Kimura et al., 1996). In this paper, we have firstly reported that IRF9 is a negative regulator of TRIF-mediated NF-κB pathway. In addition, we discovered that inhibitory effect of IRF9 primary depends on DBD domain. This study provides an evidence for the regulatory mechanisms of TRIF signaling by IRFs and enriches the content of the negative regulation of TLR signaling pathway.

## 2. Material and methods

### 2.1. Plasmids construction

The full-length CDS region of miiuy croaker (*Miichthys miiuy*) IRF9 was amplified by PCR with primers including restriction enzyme cutting sites *Kpn* I and *Xba* I with HA tag, the product was digested by corresponding enzymes and ligated into the pcDNA3.1 vector. The recombinant plasmid of TRIF from miiuy croaker was cloned into the *Kpn* I and *BamH* I sites with FLAG tag of pCDNA3.1 (Supplemental Table 1). The recombinant plasmid was confirmed by double enzyme digestion and sequencing. The four deletion mutants of IRF9,including IRF9ΔNLS1, IRF9ΔNLS2, IRF9ΔDBD and IRF9ΔIAD that deleted NLS1, NLS2, DBD and IAD domain respectively, were generated by PCR based on the IRF9 recombinant plasmid by using specific primers. And the oligonucleotide of IRF9-shRNA was designed and ligated into *BamH* I and *EcoR* I of pSIREN-RetroQZsGreen1 vector (Clontech). All of the plasmids were extracted using EndotoxinFree Plasmid DNA Miniprep Kit (Tiangen) according to the manufacturer's protocol.

### 2.2. Cell culture and transient transfections

HEK293 cells were cultured in DMEM medium which contained the 10% FBS (Fetal Bovine Serum, Gibco), 2 mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin and under humidified conditions with 5% $CO_2$ at 37 °C. Then HEK293 cells were cotransfected with NF-κB luciferase reporter gene plasmid, together with wide-type IRF9 plasmid, the four mumants of IRF9 plasmid, or TRIF expression plasmid with 0.25 μg respectively by using Lipofectamin 2000™ (Invitrogen). Renilla luciferase reporter (pRL-TK, Promega) plasmid was used as the internal control. The concentrations of plasmids were determined by Nanodrop 2000 spectrophotometer (Thermo scientific). The proportion of the amount plasmids: pRL-TK: NF-κB is 1:10. Moreover, the control group used the equal amount of corresponding empty vector compared with the experimental group.

### 2.3. Western blotting

At 48 h post-transfection, the cells were washed using ice-cold PBS. Then, the cells were lysed with 1XSDS-PAGE loading buffer and collected into centrifuge tube. Then, the samples were heated in 95 °C 5 min. In SDS-PAGE, 20 μl of the sample were load onto gel, which was electrophoretically transferred onto PVDF membranes (Pall Corporation) with a semi-dry process (Bio-Rad Trans Blot Turbo System). The membranes were blocked with 5% skim milk in TBST for 90 min and then incubated with Anti-HA mouse monoclonal antibody (Beyotime) at 4 °C overnight. 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 by using BeyoECL Plus (Beyotime) and digital imaging was performed with a cold CCD camera (Xu et al., 2016).

### 2.4. Luciferase reporter assays

At 24–48 h post-transfection, the total cell lysates were analyzed with a dual luciferase reporter assay system (Promega). In addition, the cells supplemented with 0.5 μg poly(I:C) (InvivoGen) in medium or were transfected with 0.5 μg poly(I:C) into cells at 24 h post-transfection. For an additional 12 h, cell lysates were prepared for measured. All of above experiments were performed at least triplicates.

### 2.5. qRT-PCR analysis

A 7300 RT-PCR system (Applied Biosystems, USA) using a SYBR premix ExTaq™ kit (TaKaRa) was used. Each sample was performed in triplicate for PCR amplification and PCR cycling conditions were as follows: 10 s at 95 °C, followed by 40 cycles consisting of 5 s at 95 °C, then 31 s at 60 °C.

### 2.6. Immunocytochemistry

HEK293 cells were co-transfected with 0.5 μg of each plasmid by using Lipofectamine 2000™. At 48 h post-transfection, the cells were washed with PBS and fixed with Immunol Staining Fix Solution for 20 min at room temperature. The cells were blocked with Immunol Staining Blocking Buffer and then incubated with primary antibody in Immunol Staining Blocking Buffer at 4 °C overnight and then with Cy3-labeled secondary antibody (Beyotime). The images were obtained under a fluorescence microscope (Leica).

### 2.7. Statistical analysis

Data were presented as mean ± SE. Significant differences between groups were determined by two-tailed Student's *t*-test. For all test, a *p* value of less than 0.05 was considered statistically significant.