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Nuclear Factor-κB Plays a Positive Role in TNFα Expression in Golden Pompano, *Trachinotus ovatus* (Linnaeus 1758)

*Ke-Cheng Zhu [1,2], Hua-Yang Guo [1,2], Liang Guo [1,2], Bao-Suo Liu [1,2], Nan Zhang [1,2], Shi-Gui Jiang [1,2,3,4] and Dian-Chang Zhang [1,2,3,4]**

[1] Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture and Rural Affairs; South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China, [2] Guangdong Provincial Engineer Technology Research Center of Marine Biological Seed Industry, Guangzhou, China, [3] Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya, China, [4] Sanya Tropical Fisheries Research Institute, Sanya, China

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***Correspondence:**
Dian-Chang Zhang
zhangdch@scsfri.ac.cn

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Tumour necrosis factor-α (TNFα) is a multifarious mediator of lymphoid tissue growth and antimicrobial defence mechanisms, and it acts as a pro-inflammatory regulator. The function of TNFα in parasite infection and the underlying mechanism through which nuclear factor-κB (NF-κB) regulates TNFα remain largely unclear in teleosts. In the present study, TNFα (*ToTNFα*) from golden pompano (*Trachinotus ovatus*) was identified, and its sequence features and expression levels were determined. The genomic DNA sequence is composed of 1,130 bp, consists of four exons and three introns, and encodes 341 amino acid polypeptides. The putative protein sequence shares 34.7%–61.9% identity with fish TNFα and possesses a TNF family signature and two conserved cysteine residues. Moreover, the expressions of *ToNF-κB* and *ToTNFα* are constitutively expressed in all examined tissues, with higher levels observed in the immune relevant tissues. Both *ToNF-κB* and *ToTNFα* transcription was increased in the local infection sites (skin and gill) and system immune tissues (liver, spleen and head kidney) after *Cryptocaryon irritans* stimulation. In addition, to investigate whether ToNF-κB is a regulator of *ToTNFα*, promoter analysis was performed. The region from -970 to +79 bp is known as the core promoter by different truncated mutants of *ToTNFα*. Subsequently, the activity of the *ToTNFα-p2* promoter was dramatically reduced after targeted mutation of the M6-binding site. Additionally, an electrophoretic mobile shift assay (EMSA) verified that ToNF-κB interacted with the M6-binding site in the *ToTNFα* promoter region to control the expression of *ToTNFα*. In conclusion, the present study provides the positive regulation of *TNFα* transcription by NF-κB and contributes to a better understanding of the transcriptional mechanism of *TNFα* in fish.

Keywords: *Trachinotus ovatus*, promoter activity, transcription factor, TNFα, NF-κB

INTRODUCTION

TNFα is a pleiotropic cytokine that controls pathological processes and multiple physiological processes, including the progression of chronic inflammatory diseases and cell necrosis, apoptosis, and survival (Takada et al., 2007; Chu, 2013). Both *in vivo* and *in vitro*, TNFα can regulate inflammatory cytokines, such as four interleukin (IL)-1/6/8/17C, indicating its core role in the mediation of the inflammatory response (Wollenberg et al., 1993; Amiot et al., 1997; Hong et al., 2013). Moreover, TNFα is a type-II transmembrane protein produced by multifarious cells, including natural killer, neutrophils, monocytes, macrophages, and T/B-lymphocyte cells (Tracey and Cerami, 1993; Ma, 2001; Goetz et al., 2004). In a cell-to-cell contact fashion, the biological functions of the transmembrane TNFα precursor are different from the characteristics of soluble TNFα (Horiuchi et al., 2010).

In fish, TNFα has been found in more than 20 species, such as in important seawater species, Japanese flounder (*Paralichthys olivaceus*) (Hirono et al., 2000), gilthead seabream (*Sparus aurata*) (García-Castillo et al., 2002), rainbow trout (*Oncorhynchus mykiss*) (Zou et al., 2002; Hong et al., 2013), catfish (*Ictalurus punctatus*) (Zou et al., 2003), turbot (*Psetta maxima*) (Ordás et al., 2007), sea bass (*Dicentrarchus labrax*) (Nascimento et al., 2007), bluefin tuna (*Thunnus orientalis*) (Kadowaki et al., 2009), striped trumpeter (*Latris lineata*) (Covello et al., 2009), orange-spotted grouper (*Epinephelus coioides*) (Lam et al., 2011), rock bream (*Oplegnathus fasciatus*) (Kim et al., 2011; Ko et al., 2022), fugu (*Fugu rubripes*) (Biswas et al., 2015), large yellow croaker (*Larimichthys crocea*) (Huang et al., 2020), and pufferfish (*Takifugu obscurus*) (Kong et al., 2021), and in important freshwater species, common carp (*Cyprinus carpio*) (Forlenza et al., 2009; Zhao et al., 2012), zebrafish (*Danio rerio*) (Savan et al., 2005), ayu (*Plecoglossus altivelis*) (Uenobe et al., 2007), goldfish (*Carassius auratus*) (Grayfer et al., 2008), grass carp (*Ctenopharyngodon idella*) (Zhang et al., 2012), and snakehead (*Channa argus*) (Cui et al., 2020). Furthermore, two TNFα isoforms have been characterized in Atlantic bluefin tuna *Thunnus thynnus* (Lepen Pleić et al., 2014), *T. orientalis* (Kadowaki et al., 2009), *E. coioides* (Lam et al., 2011), and *C. auratus* (Grayfer et al., 2008), three TNFα isoforms in *O. mykiss* (Hong et al., 2013), and even four in *C. carpio* (Savan and Sakai, 2004; Zhao et al., 2012). Moreover, all fish *TNFα* molecules possess typical structures of the TNF family, containing a TACE restriction site at position S^{71}/L^{72}, a transmembrane domain, a TNF family signature (I^{118}-F^{131}) (Cui et al., 2020; Huang et al., 2020), and two conserved cysteine residues (C^{39} and C^{179}) that are essential for disulphide bridges in the tertiary structure of TNFα (Savan et al., 2005). Nevertheless, another gene in the *TNF-β* family, which also exists in mammals, shares 50% homology at the protein level (Bodmer et al., 2002).

The transcription factor nuclear factor-κB (NF-κB) includes several subunits, such as RelA, RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100), which are evolutionarily conserved and play an important role in the regulation of numerous basic cellular processes (Cohen et al., 2006; Wang et al., 2013). It can be induced by various stimuli, such as cytokines, growth factors, mitogens, and viral/bacterial pathogens (Kravtsova-Ivantsiv et al., 2009; Lapid et al., 2017). Furthermore, some research has shown that apoptosis is inhibited, and *TNFα* is activated in HEK293T cells after overexpression of the sea cucumber *Holothuria leucospilota NF-κB* (He et al., 2021). Consequently, to confirm whether a similar regulatory relationship exists in golden pompano *Trachinotus ovatus*, the sequence characterization, expression pattern, and transcriptional regulation of *ToTNFα* by ToNF-κB (p105) were determined. The present study examining ToNF-κB (p105) will be useful for tracing the TNFα system in marine fishes and promoting the development of the anti-parasite industry in the future.

MATERIALS AND METHODS

Cryptocaryon irritans Challenge and Sampling

Healthy golden pompano (98 ± 15 g), purchased from the Linshui Marine Fish Farm of Hainan Province, China, were maintained in a fresh seawater system at 28 ± 2°C, accompanied by 25‰ salinity. The fish were allowed to feed on commercial feed (crude protein and crude fat >37% and >7%, respectively, Hengxin, Zhanjiang, China) for 2 weeks before the experiment. In Lingshui Marine Fish Farm, no disease outbreaks was recorded during the course of breeding. The gills and mucus from 10 fish were observed using a light microscope to ensure there were no *C. irritans* theronts, and their serum was tested for the presence or absence of parasite infection.

One hundred and twenty healthy pompano were challenged with *C. irritans* theronts at a dose of 600 theronts/fish according to previous research (Dan et al., 2006; Zhu et al., 2020). Fifty fish were known as the control group. The skin, gill, liver, spleen, and head kidney were collected from six fish after 0 h, 6 h, 12 h, 1 day, 2 days, and 3 days of infection. The same tissues from uninfected pompano were defined as the negative control at each time point. Three replicates were collected at each time point. We used MS222 (0.1 g/l; Sigma, Alcobendas, Spain) to narcotize the pompano before dissection. All samples were immediately frozen in liquid nitrogen and then stored at -80°C until use.

RNA Extraction and Gene Cloning

A mortar and pestle were used to homogenize the tissues, accompanied by addition into liquid nitrogen. Then, total RNA (1 μg) was isolated from each sample with the HiPure Fibrous RNA Plus Kit (Magen, Guangzhou, China) according to the manufacturer's protocol and stored at -80°C. cDNA was synthesized by Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Plasmid Construction, Cell Culture and Dual-Luciferase Reporter Assays

Total genomic DNA was isolated from *T. ovatus* muscle and is known as a template for augmenting alternative promoter sequences (Sun et al., 2013). To confirm the interaction effect of *ToTNFα* with ToNF-κB, five truncated mutants from the *ToTNFα* promoter were amplified by specific primers with *XhoI* and *KpnI* restriction sites (**Table 1**). Subsequently, the five truncated fragments [denoted pGL3-basic-TNFα-p1 (-1,969 to +79), pGL3-basic-TNFα-p2 (-970 to +79), pGL3-basic-TNFα-p3 (-736 to +79), pGL3-basic-TNFα-p4 (-446 to +79) and pGL3-basic-TNFα-p5 (-150 to +79)] were subcloned into the pGL3-basic luciferase reporter plasmid (Promega, Madison, WI, USA) with the corresponding restriction sites.

To validate the potential role of the ToNF-κB binding sites on the core *ToTNFα* promoter, six assumed recombinant plasmids of mutations were constructed. To predict the underlying binding sites of ToNF-κB on the *ToTNFα* promoter, we used the transcription factor-binding site prediction (TFBS)-JASPAR database (http://jaspar.genereg.net/), TRANSFAC®, and MatInspector® software. Moreover, according to the manufacturer's protocol, truncated mutants of the pGL3-basic-*TNFα-P2* promoter were generated by a Muta-Direct™ site-directed mutagenesis kit (SBS Genetech, Shanghai, China). The pGL3-basic-TNFα-P2 promoter was considered wild-type. To acquire the six mutants, the procedure of PCR augmentation was applied as in a previous study (Dong et al., 2016). The six point mutants directly deleted the prediction of six binding sites, which included M1 (-804 to -783 bp), M2 (-682 to -661 bp), M3 (-530 to -509 bp), M4 (-433 to -412 bp), M5 (-197 to -176 bp) and M6 (-116 to -92 bp) from the wild-type promoter.

TABLE 1 | Primers used for sequence cloning, deletion mutant construction, mRNA construction and qRT-PCR.

Subject and primers	Nucleotide sequence
Primers for sequence cloning	
TNFα-ORF-F	ATGGAGGGTGAATGTAAAGTGC
TNFα-ORF-R	TCACAGTGCAAACACACCA
NF-κB-ORF-F	ATGGCTGGAGACGACCACTA
NF-κB-ORF-R	TTACGCTGTGGAAAGCTC
Deletion mutant construction	
TNFα-pF1	CCGCTCGAGCAGAGTGTGTGCTGCTCTCA
TNFα-pF2	CCGCTCGAGATTAACGTTTGTTTTCGTTG
TNFα-pF3	CCGCTCGAGTGTCACCATGAAGACATTC
TNFα-pF4	CCGCTCGAGGTTACCCCACACTTTGACGT
TNFα-pF5	CCGCTCGAGGCCATTTGTGGGAGTGTGTG
TNFα-pR	CGGGGTACCTGTTTGACACCCTGCGTGGT
Primers for qRT-PCR	
qRT-TNFα-F	GGAGGGTGAATGTAAAGTGC
qRT-TNFα-R	CCGTGGTTAGTTTTGAGTTGT
qRT-NF-κB-F	ACTACCAACCCGATTTTTGACAT
qRT-NF-κB -R	GACGGACCTTCGCAACCAT
EF1α-F	AAGCCAGGTATGGTTGTCAACTTT
EF1α-R	CGTGGTGCATCTCCACAGACT
EMSA assays	
TNFα-MUT	GAACAGCACTGTCAGGACCTCTTTC
TNFα-WT	GGGTGACATCACTGGAGTTTCCCTC

Underline word indicate restriction enzyme cutting site

The homologous TF-binding site sequences are shown in **Figure 1A**.

The Renilla luciferase plasmid pRL-TK (Promega, USA) was used as an internal control. The TransGen Plasmid Mini Kit (Beijing, China) was used to isolate recombinant plasmids. Human embryonic kidney (HEK293T) (GeneCreate, Wuhan, China) cell cultures and transfection experiments were performed according to the methods described by Li et al. (2017). To verify whether ToNF-κB could upregulate *ToTNFα in vitro*, HEK293T cells overexpressing pcDNA3.1-NF-κB and pcDNA3.1-Flag were used.

Electrophoretic Mobility Shift Assay

The EMSA experimental method was performed according to a previously described procedure (Yu et al., 2010). Briefly, for DNA/protein conjugation reactions, the lysates of HEK293T cells transfected with pcDNA3.1-Flag-NF-κB were provided. To mark the wild-type and mutated oligonucleotides (**Table S2**), the EMSA Probe Biotin Labelling Kit (Beyotime, Shanghai, China) was used according to the manufacturer's instructions. DNA/protein-binding reactions were accomplished using an EMSA/Gel-Shift Kit (Beyotime, China) at 25°C. To exclusively understand the DNA/protein-binding reactions, competition assays were implemented with 100× excessive non-marked mutated or wild-type probes. Thus, the completed reactions were divided on non-denaturing 4% PAGE gels for 20 min. A LightShift® Chemiluminescent EMSA Kit (Pierce, Waltham, MA, USA) was used to develop the proteins by the autoradiography method.

Quantitative Real-Time PCR and Statistical Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyse the expression patterns of *ToNF-κB* and *ToTNFα* in five infected tissues (skin, gill, liver, spleen, head kidney) from *T. ovatus*. The specific primers for *ToNF-κB* and *ToTNFα* and the housekeeping gene *EF-1α* (elongation factor 1, alpha) are shown in **Table 1**. The qRT-PCR procedure was executed as previously described (Zhang et al., 2018). To evaluate the relative expression of those genes, the $2^{-\Delta\Delta CT}$ method was used (Livak and Schmittgen, 2001). The data from different tissues and groups were analysed by the Duncan test using one-way ANOVA. Data are presented as the means of three replicates ± SD, and the level of statistical significance was set at $p < 0.05$.

RESULTS

Sequence Characterization of *ToTNFα*

The open reading frame (ORF) of *ToTNFα* (GenBank accession number: OM777150, **Figure 2**) was 726 bp with a predicted theoretical isoelectric point (PI) of 6.13 and a molecular weight (Mw) of 27.18 kDa. Similar to most fish TNFα molecules, ToTNFα has a TACE restriction site at position S^{71}/L^{72} and a TNF family signature (I^{118}-F^{131}; **Figure 2**

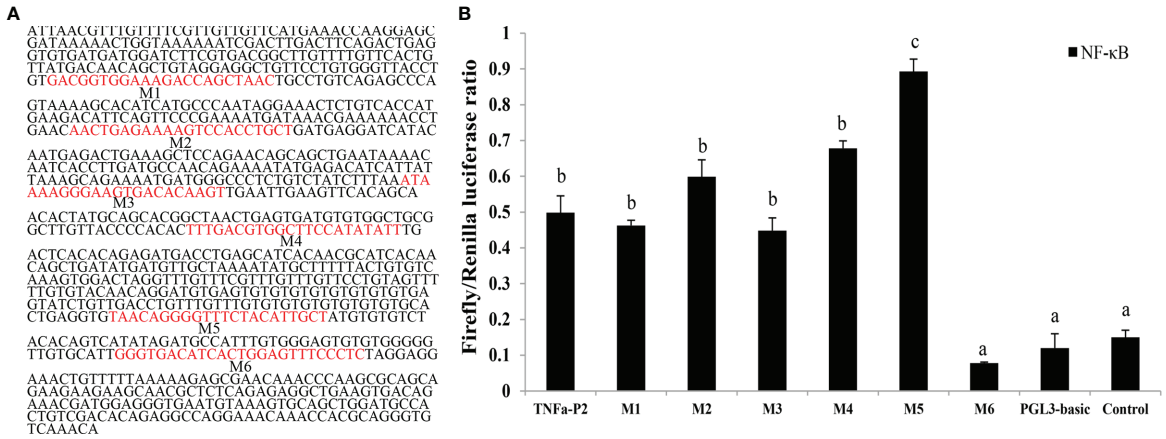

FIGURE 1 | Construction of truncated mutants for the identification of predicted transcription factor (TF) binding sites in the *ToTNFα* promoter. **(A)** The nucleotide sequence and predicted binding sites in the core region of the *ToTNFα* promoter. **(B)** Effects of three mutants on *To TNFα-P2* promoter activity. Binding sites are shown with boxes. Mutations of promoter sequences are listed in **Table 2**. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences ($p < 0.05$).

TABLE 2 | Primers used for site-directed mutations of putative binding sites on *ToTNFα* promoter.

Putative binding sites	Nucleotide sequence	Mutated pattern
M1	GACGGTGGAAAGACCAGCTAAC	Deletion
M2	AACTGAGAAAAGTCCACCTGCT	Deletion
M3	ATAAAAGGGAAGTGACACAAGT	Deletion
M4	TTTGACGTGGCTTCCATATATT	Deletion
M5	TAACAGGGGTTTCTACATTGCT	Deletion
M6	GGGTGACATCACTGGAGTTTCCCTC	Deletion

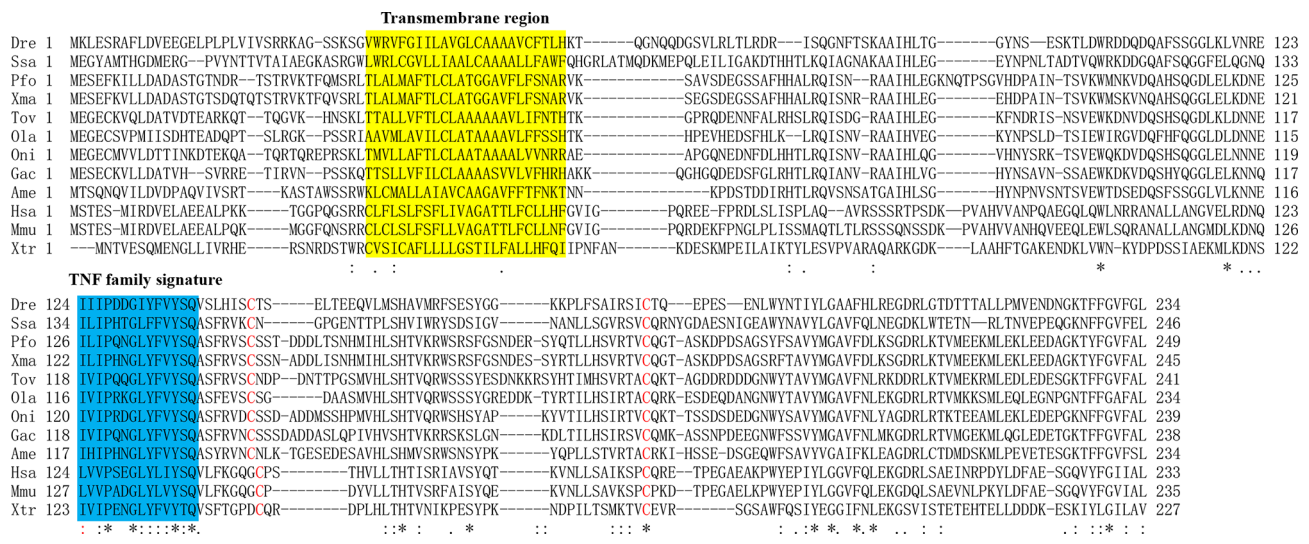

FIGURE 2 | Alignments of TNF*α* amino acid sequences from different species. The TNF signature motif is shown in the blue box. The transmembrane region is shown in the yellow box. Cysteines conserved in TNF*α* are marked by red. Identical (asterisks) and similar (: or.) residues identified by the CLUSTAL W program are indicated. The Latin abbreviation and accession numbers are listed in **Table S1**.

and **Figure S1**). Two conserved cysteine residues, C^{138} and C^{183}, are found in ToTNFα sequences; C^{138} is only conserved in teleost fish TNFα, but C^{183} exists in all known species TNFα (**Figure 2** and **Figure S1**). The assumed total aa sequence of ToTNFα shares higher identity with *Oreochromis niloticus* TNFα (61.9%), *Oryzias latipes* TNFα (59.3%), *Gasterosteus aculeatus* TNFα (58.8%), *Poecilia formosa* TNFα (56.2%), while it has only 25.6% identity to *Homo sapiens* TNFα1 (**Table S1**). Moreover, the ORF of *ToNF-κB* (GenBank accession number: OM777151, **Figure S2**) was 2,826 bp with a predicted theoretical isoelectric point (PI) of 4.84 and a molecular weight (Mw) of 100.98 kDa.

ToTNFα Structural and Phylogenetic Analysis

The genomic sequence of *ToTNFα* is 1,130 bp, containing four exons and three introns (**Figure 3** and **Figure S3**). All the 5′/3′ ends of the introns displayed typical sequence characteristics (GT/introns/AG). The distributions and lengths of the introns and exons of metazoan *TNFα* genes are shown in **Table S2**.

Additionally, the phylogenetic relationship of TNFα has been determined in vertebrates (**Figure 3**). The computer program Clustal W alignment and MEGA 6.0 software were used to construct a phylogenetic tree of TNFα aa sequences with a maximum likelihood (ML) method. In the phylogenetic tree, ToTNFα is grouped together with *O. niloticus* and *G. aculeatus*, and the homology with ToTNFα, from close to distant, is other Osteichthyes, Amphibia, Aves and Mammalia (**Figure 3**). This result was consistent with the traditional taxonomic relationship of the above species.

Tissue Expression of *ToNF-κB* and *ToTNFα*

To confirm the role of *ToNF-κB* and *ToTNFα* in various tissues, the constitutive expression of two genes in gill, head-kidney, brain, small intestine, spleen, fin, liver, white muscle, stomach, blood and male and female gonads was detected by qRT-PCR (**Figure 4**). The highest expression level of *ToNF-κB* was in the gill, head-kidney, skin and small intestine, while a low expression was observed in the liver and white muscle ($p < 0.05$) (**Figure 4A**). Moreover, the highest expression level of *ToTNFα* was also in the head-kidney, gill, skin and small intestine, while a low expression was observed in the stomach, liver and blood ($p < 0.05$) (**Figure 4B**).

To analyse the possible role of *ToNF-κB* and *ToTNFα* in defence against parasite infection, the mRNA levels of *ToNF-κB* (**Figure 5A**) and *ToTNFα* (**Figure 5B**) were determined in local infection sites (skin and gills) and system immune tissue (liver, spleen and head kidney) after *C. irritans* challenge.

The expression of *ToNF-κB* was upregulated in those five tissues after *C. irritans* stimulation. In the gill, *ToNF-κB* was upregulated at 3–12 h, and the peak of expression (2.49-fold relative to the uninfected control) was observed at 12 h. In the skin, *ToNF-κB* was increased from 3 to 12 h, with the peak of expression at 12 h, which was 31.9-fold relative to the uninfected control. In the liver, *ToNF-κB* was upregulated at 3 h, 6 h, 12 h and 1 day and then returned to normal levels. In the spleen, *ToNF-κB* was upregulated from 3 to 6 h and then returned to normal levels. In the head kidney, the peak of *ToNF-κB* expression was 2.49-fold relative to the uninfected control at 12 h (**Figure 5A**). Moreover, after *C. irritans* induction, the expression of *ToTNFα* was also increased in those five tissues. In the gill, *ToTNFα* was upregulated at 3 and 6 h and then returned to normal levels. There was also a second peak of *ToTNFα*

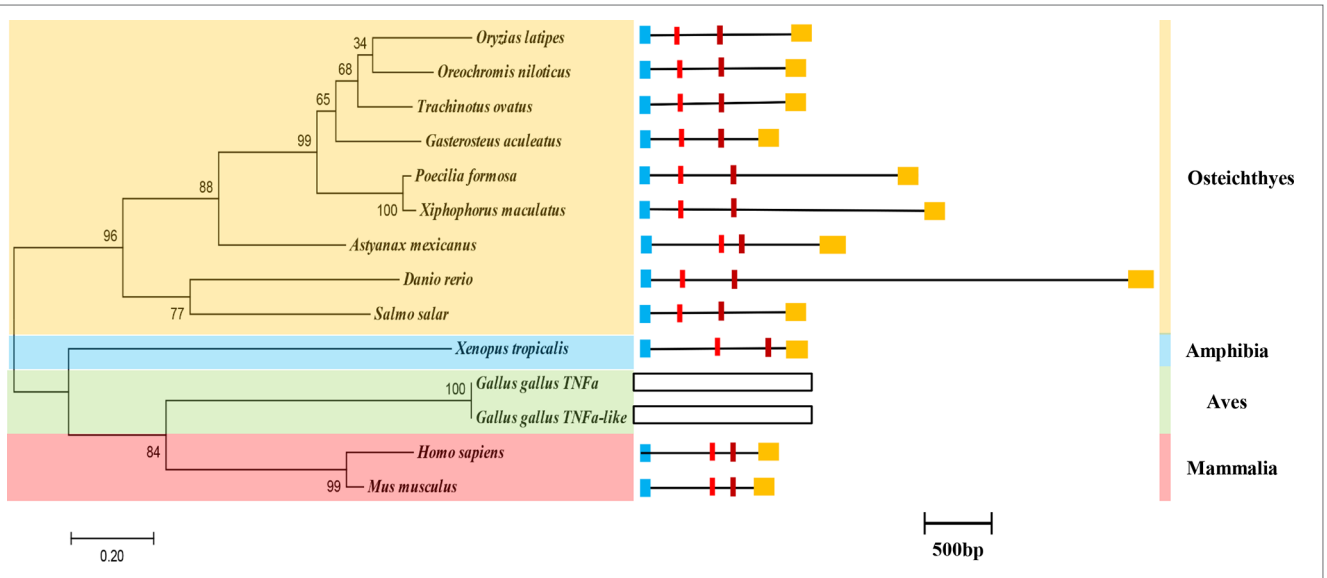

FIGURE 3 | The phylogenetic relationship and genome structure of the *ToTNFα* gene. Genome structure analysis of *ToTNFα* genes according to their phylogenetic relationship. Lengths of exons and introns of each *TNFα* gene are displayed proportionally. Different colour boxes and lines represent exons and introns, respectively. The identical colour boxes represent homologous sequences.

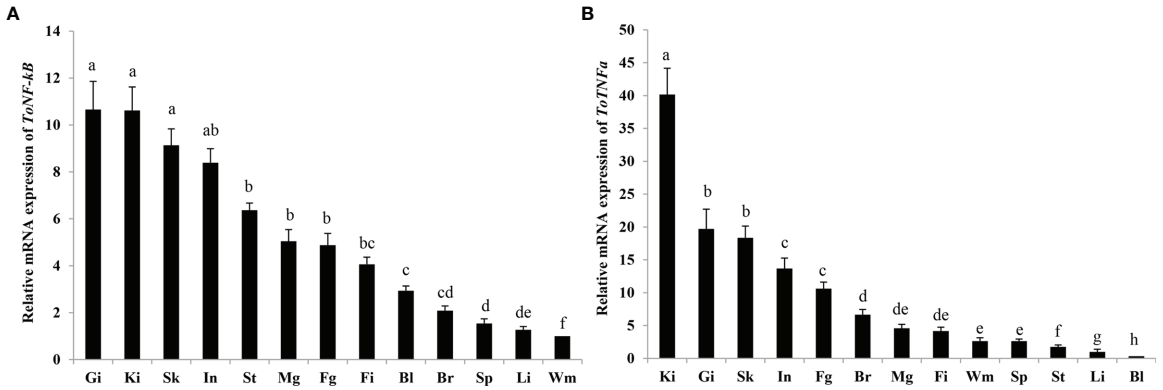

FIGURE 4 | Expression patterns of *ToNF-κB* **(A)** and *ToTNFα* **(B)** gene transcripts in various tissues. The 12 tissues are skin (Sk), brain (Br), blood (Bl), gill (Gi), fin (Fi), head-kidney (Ki), small intestine (In), stomach (St), spleen (Sp), liver (Li), white muscle (Wm), female gonad (Fg), and male gonad (Mg). Different letters indicate significant differences ($p < 0.05$).

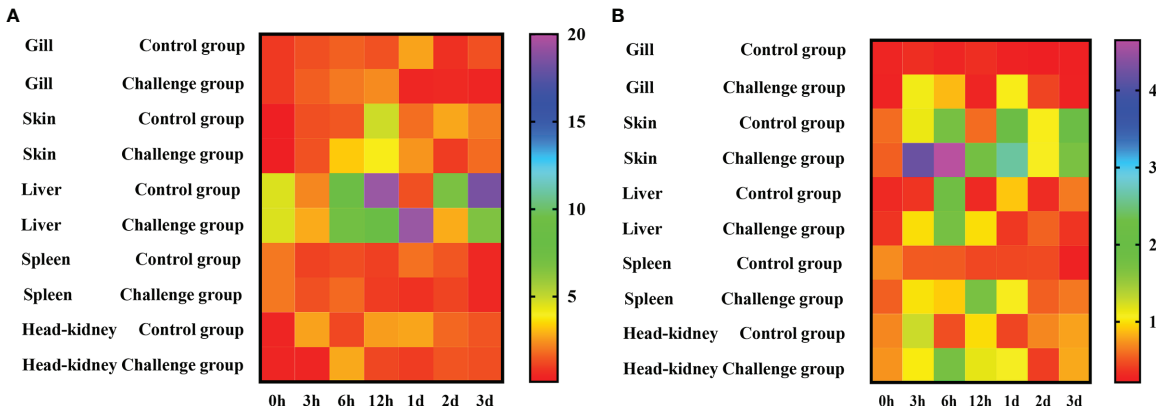

FIGURE 5 | Temporal expression of *ToNF-κB* **(A)** and *ToTNFα* **(B)** mRNA is detected in gill, skin, liver, spleen, and kidney by qRT-PCR after infection with *C. irritans* (0, 3, 6, 12 hpi, 1 day, 2 days, 3 days). EF-1a is used as the internal control to calibrate the cDNA templates for all the samples. The heatmap is constructed by GraphPad Prism software.

expression at day 1, which then returned to normal levels. In the skin, *ToTNFα* was upregulated from 3 h to 1 day, and the peak of expression (7.74-fold relative to the uninfected control) was observed at 6 h. In the spleen, *ToTNFα* was upregulated from 3 h to 1 day and then dramatically decreased at 2 and 3 days. In the head kidney, *ToTNFα* expression was significantly increased at 6 h and 1 day, with the peak expression (2.29-fold relative to the uninfected control) at 6 h (**Figure 5B**). *ToTNFα* was upregulated at early time points in both systemic immune tissues and local infection sites, implying that *ToTNFα* might be involved in host defence against *C. irritans* by a combination of systemic and mucosal immunity.

ToNF-κB Promotes *ToTNFα* Expression

A total of 2,048 bp of the 5′-flanking sequence of the *ToTNFα* gene was obtained and identified as the candidate promoter. To investigate the promoter activity of *ToTNFα* in response to ToNF-κB in HEK293T cells, a series of truncated mutants, which included pGL3-basic-TNFα-p1, pGL3-basic-TNFα-p2, pGL3-basic-TNFα-p3, pGL3-basic-TNFα-p4 and pGL3-basic-TNFα-p5, were constructed (**Figure 6A**). The expression level of *TNFα-P2* was higher than the activity of other mutants with the ToNF-κB response (**Figure 6B**), suggesting that the centre promoter region was present between -970 and +79 bp, which contained the NF-κB-binding sites.

To further pinpoint the ToNF-κB-binding sites in the *ToTNFα* promoter, the binding sites were predicted and mutated (**Figure 1A**; **Table 2**). Exogenous cells were co-transfected with ToNF-κB and mutant vectors (M1, M2, M3, M4, M5 or M6) or the empty vector (pGL3-basic). Notably, the results indicated that only mutations of the M5 (-197 to -176 bp) and M6 (-116 to -92 bp) binding sites caused dramatic increases and reductions in promoter activity (**Figure 1B**), respectively. Furthermore, no significant difference was found between the wild type (TNFα-p2) and M1, M2, M3 or M4. It was suggested

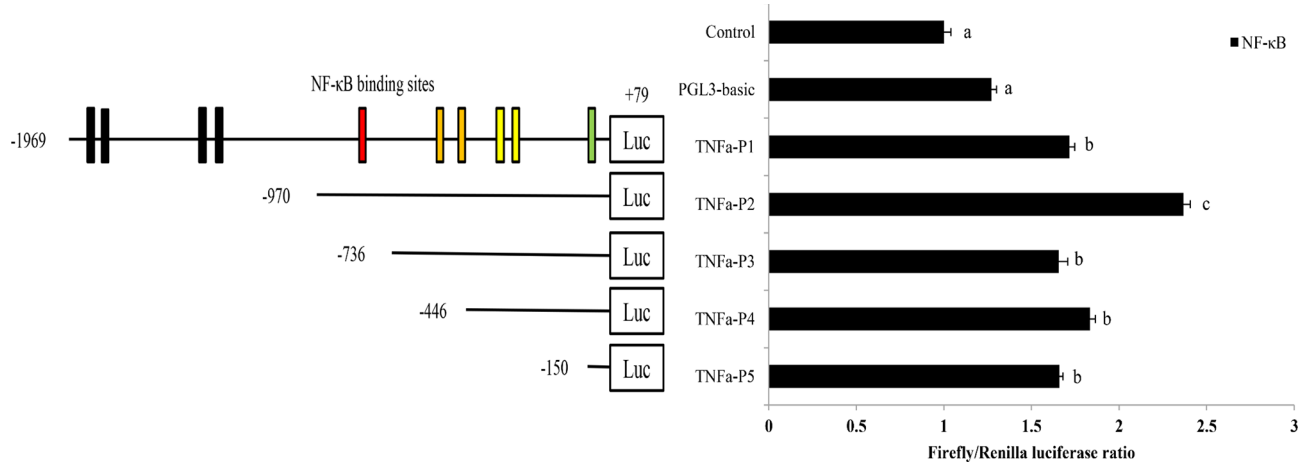

FIGURE 6 | Promoter activity analysis of the *ToTNFα* gene. The structure and transcriptional activity of the *ToTNFα* promoter. Five recombinant plasmids, denoted TNF*α*-P1 (-1,969 to +79), TNF*α*-P2 (-970 to +79), TNF*α*-P3 (-736 to +79), TNF*α*-P4 (-446 to +79) and TNF*α*-P5 (-150 to +79), were constructed and transfected, along with the transcription factor ToNF-κB, into HEK 293T cells. Dual-luciferase activity was driven by the *ToTNFα* promoter upon the transfection of pcDNA3.1-NF-κB or pcDNA3.1 into HEK 293T cells. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences ($p < 0.05$).

that the M5 mutation site in the *ToTNFα-P2* promoter was an important site for triggering *ToTNFα*-upregulated expression by ToNF-κB.

To further confirm the ToNF-κB-binding motif in the *ToTNFα* promoter, an EMSA was performed. Based on the predicted ToNF-κB-binding site, oligonucleotide probes were synthesized (**Table S2**) and incubated with HEK293T cell lysates, including recombinant NF-κB, *in vitro*. Recombinant NF-κB bound to the oligonucleotide probes of the predicted NF-κB-binding site in the *ToTNFα* promoter. Nevertheless, mutations in the NF-κB-binding site resulted in the dissociation of the DNA-rNF-κB complex (**Figure 7**), suggesting that NF-κB specifically interacted with the M5 motif in the *ToTNFα* promoter. The formation of the DNA-rNF-κB complex was specific, as it could only be blocked by excessive unlabelled control probes (100×).

DISCUSSION

A member of this family, *TNFa*, is a major mediator of proinflammatory and antimicrobial defence mechanisms and is able to eliminate various pathogens by inducing a variety of cellular responses, such as phagocytosis and chemotaxis, and is considered an excellent biomarker and health indicator for both mammals and fish (Frederick et al., 2004; Kohchi et al., 2006). Indeed, *NF-κB* plays an important role in the regulation of *TNFα* pathway genes. In the present study, to further comprehend the molecular level of TNFα in teleosts, we first characterized TNFα from *T. ovatus*, with particular attention to the genomic structure, expression levels and regulation by NF-κB.

Similar to other TNFAs in vertebrates, the deduced aa sequence of ToTNFα contained a TACE restriction site at position S^{71}/L^{72} and a TNF family signature (I^{118}-F^{131}; **Figure 2** and **Figure S1**) (Cui et al., 2020; Huang et al., 2020). Two conserved cysteine residues, one of which is only conserved in teleost fish TNFα and the other of which exists in all known species TNFα. Therefore, conserved structural domains in fish TNFα molecules may suggest their functional similarity. Moreover, the assumed total aa sequence of ToTNFα shares higher identity with *O. niloticus* TNFα (61.9%), *O. latipes* TNFα (59.3%) and *G. aculeatus* TNFα (58.8%), which is analogous to the result of phylogenetic analysis. The evolutionary relationship was in accordance with the findings of conventional taxonomy, showing that *ToTNFα* displayed a close genetic relationship with Perciformes. Furthermore, similar to the *TNFα* gene in all detected vertebrates, the genomic sequence of *ToTNFα* is also composed of four exons and three introns. These results

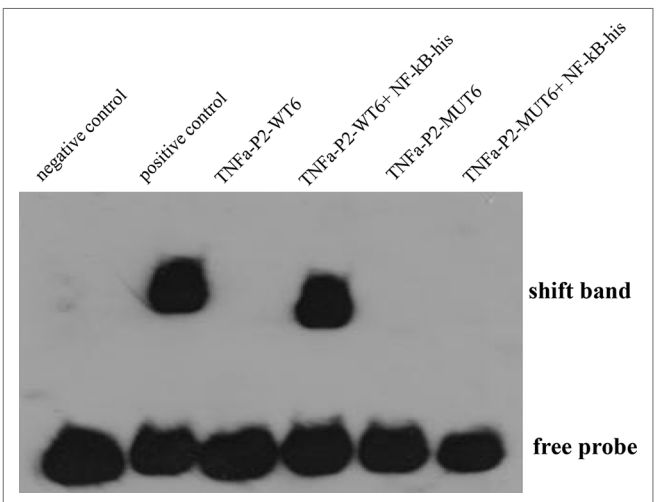

FIGURE 7 | Binding reactions of ToNF-κB and the *ToTNFα* promoter. Biotin-labelled EMSA probes are incubated with lysates of HEK293T cells containing NF-κB protein. WT, wild-type probe; MUT, mutated probe. Positive control indicates ToTNFα+Protein; negative control indicates ToTNF*α*-WT.

indicated that the genomic structure of vertebrate TNFαs was evolutionarily conserved.

In the present study, *ToTNFα* transcripts were constitutively expressed in all tested tissues of healthy fish, which was similar to the expression of *TNFα* in other teleosts (Hirono et al., 2000; García-Castillo et al., 2002; Zou et al., 2003; Savan and Sakai, 2004; Hong et al., 2013; Biswas et al., 2015; Cui et al., 2020; Huang et al., 2020; Kong et al., 2021; Ko et al., 2022). The mRNA level of *ToTNFα* was rich in the head-kidney, gill, skin and small intestine and poor in the stomach, liver and blood. Similarly, the highest expression levels of trout *TNFα1/2/3* (Hong et al., 2013) and *Thunnus orientalists TNFα2* (Kadowaki et al., 2009) were also determined in the gill. Therefore, a high expression of *TNFα* exists in immune tissues. Moreover, both *TNFα1* and *TNFα2* transcripts also had the lowest expression in the stomach (Huang et al., 2020). The reason for the diversity in *TNFα* expression patterns in different teleosts might be due to the multiple immune systems of fishes (Lepen Pleić et al., 2014). Additionally, the *ToNF-κB* transcript was found to be widely expressed in all tested tissues of healthy fish. It is likely that *ToNF-κB* is an important transcription factor (He et al., 2021). *ToNF-κB* expression was highest in the intestine, suggesting that the intestine is an important tissue associated with the immune defence response to various stresses in pompanos (Zhang et al., 2018; He et al., 2021).

Previous studies have declared that viruses [grass carp reovirus (GCRV)], poly I: C, and bacteria (LPS, *Vibrio alginolyticus*, *Photobacterium damselae*, *Aeromonas schubertii*, *A. hydrophila*, *Nocardia seriolae*) and peptidoglycan can generate antiviral and antibacterial responses in teleosts, where *NF-κB* and *TNFα* play a role (Nascimento et al., 2007; Wang et al., 2013; Huang et al., 2020; He et al., 2021; Kong et al., 2021). Nevertheless, in fish, few studies have been performed to explain the role of *TNFα* in response to parasite stimulation. Moreover, *C. irritans* is one of the major threats to *T. ovatus* (Dan et al., 2006). Consequently, to analyse the possible role of *ToTNFα* in the defence against parasite infection, the mRNA level of *ToTNFα* was determined in local infection sites (skin and gills) and system immune tissues (liver, spleen and head-kidney) after *C. irritans* challenge. In our previous study, *IRF2* was upregulated at early time points in both systemic immune tissues and local infection sites in *T. ovatus* (Zhu et al., 2020). Overexpressing *ToIRF2 in vitro* notably increased the transcription of several type II IFN/IRF-based signalling pathway genes (Zhu et al., 2020). In the present study, both *ToNF-κB* and *ToTNFα* expression was upregulated in those five tissues after *C. irritans* stimulation, implying that *ToNF-κB* and *ToTNFα* might be involved in host defence against *C. irritans* by a combination of systemic and mucosal immunity. To some extent, this finding was consistent with findings in *E. coioides* and *T. ovatus* (Lam et al., 2013; Zhu et al., 2020).

Overexpression of *H. leucospilota NF-κB* could increase *TNFα* expression (He et al., 2021). In *T. ovatus*, it remains unclear whether *ToNF-κB* regulates the transcription of *ToTNFα*. To determine the promoter activity of *ToTNFα* in response to the transcription factor ToNF-κB in HEK293T cells, a series of progressive deletion constructs provided by our laboratory were used in the present study. The results of the dual-luciferase activity assay showed that the promoter activity of TNFα-P2 (-970 to +79 bp) was highest among the five plasmids in response to ToNF-κB, suggesting that the region from -970 to +79 bp contained the core promoter. Moreover, the expression levels of TNFα-P2 were 2.37-fold higher than those of the empty vector (pGL3-basic) in response to ToNF-κB. These results indicated that the core promoter region, which included the NF-κB binding sites, requires further analysis.

To further elucidate the binding of ToNF-κB to the *ToTNFα* promoter sequence, point mutations and EMSAs were analysed. In the present study, deletion of the TNFα-p2 M6 (-116 to -92 bp) binding site prominently reduced the promoter activity of *ToTNFα*. The EMSA also showed that ToNF-κB specifically bound to the *ToTNFα* promoter at the binding M6 site. ToNF-κB could bind a motif (GGGTGACATCACTGGAGTTTCCCTC) but not a mutant motif (GAACAGCACTGTCAGGACCTCTTTC) *in vitro*. Briefly, ToNF-κB could control *ToTNFα* expression by binding the M6-binding sites in fish. This study could provide basic information for the study of *TNFα* promoters in marine fish.

CONCLUSION

The expression characteristics and regulatory function of *ToNF-κB* and *ToTNFα* were reported. We verified that *ToNF-κB* and *ToTNFα* transcripts were increased in both local infection sites (skin and gill) and system immune tissues (liver, spleen and head-kidney) after challenge with *C. irritans*. Moreover, the positive regulatory function of *ToNF-κB* on *ToTNFα* expression was also determined. Thus, a positive feedback mechanism mediated by TNFα-induced *ToNF-κB* activation was proposed in *T. ovatus*. Our findings might help further understand the regulatory functions of *TNFα* in fish.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

AUTHOR CONTRIBUTIONS

S-GJ and D-CZ contributed to the conception and design of the study. K-CZ and B-SL organized the database. H-YG and LG performed the experiments. B-SL and NZ performed the bioinformatics and statistical analysis. K-CZ wrote the

manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.884892/full#supplementary-material

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