Activation of cytokine expression occurs through the TNFα/NF-κB-mediated pathway in birnavirus-infected cells

Wei-Lun Wang, a,b Wangta Liu a, Hong-Yi Gong c, Jiann-Ruey Hong d, Ching-Chun Lin a, Jen-Leih Wu a,b,*

* Institute of Cellular and Organismic Biology, Academia Sinica, Nankang 115, Taipei, Taiwan
† Institute of Fisheries Science, National Taiwan University, Taipei, 106, Taiwan
‡ Department of Aquaculture, National Taiwan Ocean University, Keelung, 202, Taiwan
§ Institute of Biotechnology, National Cheng Kung University, Tainan 701, Taiwan

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ABSTRACT
The infectious pancreatic necrosis virus (IPNV) belongs to the Birnaviridae family of viruses and causes acute contagious diseases in a number of economically important freshwater and marine fish. In this study, we infected zebrafish embryonic cells (ZF4) with IPNV and analyzed the gene expression patterns of normal and infected cells using quantitative real-time PCR. We identified a number of immune response genes, including ifna, ifng, mx, irf1, irf2, irf4, tnfa, tnfβ, il-1b, il-15, il-26, ccl4 and mmp family genes, that are induced after viral infection. Transcriptional regulators, including cebpb, jund, nfkβ and stat1, stat4 and stat5, were also upregulated in IPNV-infected cells. In addition, we used Pathway Studio software to identify TNFα as having the greatest downstream influence among these altered genes. Treating virus-infected cells with an siRNA targeting TNFα inhibited NF-κB expression. To further interrupt the TNFα/NF-κB-mediated pathway, the expression levels of cytokines and metalloproteinases were inhibited in IPNV-infected cells. These data suggest that, during IPNV infection, the expression of cytokines and metalloproteinases might be initiated through the TNFα/NF-κB-mediated pathway. The modulation of TNFα/NF-κB-related mechanisms may provide a therapeutic strategy for inhibiting viral infection in teleosts.

1. Introduction

The infectious pancreatic necrosis virus (IPNV) is an economically important fish pathogen that causes acute infection in salmonid fry and Atlantic salmon post-smolts [1,2]. Severe necrosis has been observed in pancreatic and liver tissues in virus-infected Atlantic salmon fry [3]. IPNV is a member of the genus Aquabirnavirus in the Birnaviridae family of viruses and possess a bi-segmented, double-stranded RNA genome. The expression of viral genes requires the production of four unrelated major genes that undergo various post-translational cleavage processes to generate between three and five structural proteins [4]. The larger genome segment A encodes VP2 (major capsid; 46 kDa), VP3 (submajor capsid and pro-apoptotic protein; 32 kDa) [5], VP4 (protease; 28 kDa) and VP5 (anti-apoptotic protein; 17 kDa) [6]. The smaller segment B encodes the VP1 polypeptide (90–110 kDa), which is an RNA-dependent RNA polymerase. Previous studies have shown that an atypical form of apoptosis is induced by IPNV infection [7,8]. The expression level of the anti-apoptotic Bcl-2 gene Mcl-1 has been shown to be downregulated following IPNV infection [9]. The initiator caspase 8 and effector caspase 3 are activated and promote host cell apoptosis during IPNV pathogenesis [10]. The apoptosis induced by IPNV occurs via a viral receptor that triggers the expression of the death gene Bad, possibly through a tyrosine kinase-dependent pathway [11].

**Abbreviations**: IPNV, infectious pancreatic necrosis virus; TNFα, tumor necrosis factor alpha; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IFN, interferon; IRF, interferon regulatory factor; CCL, chemokine (C-C motif) ligand; IL, interleukin; MMP, matrix metallopeptidase; CEBP, CCAAT-enhancer-binding proteins; STAT, signal transducers and activator of transcription; siRNA, short interfering RNA; CHSE, Chinook salmon embryonic; ISG, interferon stimulated genes; MOI, multiplicity of infection; TCID, tissue culture infectious dose; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; EF1α, elongation factor 1-alpha; irgq2, immunity-related GTPase family gq2; ig15, Isg15 ubiquitin-like modifier; irfd2, interferon-related developmental regulator 2; irf2p2, interferon regulatory factor 2 binding protein 2; itgα5, integrin alpha 5; pparq, peroxisome proliferator activated receptor gamma; gata1, GATA binding protein 1a.

* Corresponding author. Current address: Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, 128 Sec. 2, Academia Rd, Nankang, Taipei 115 Taiwan. Tel.: +886 2 27899568; fax: +886 2 27824595.
E-mail address: jlwu@gate.sinica.edu.tw (J.-L. Wu).

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Zebrafish have been established as a powerful vertebrate model organism for immunological research. Previous studies have shown that zebrafish possess both innate and adaptive immune mechanisms against microbial agents [12,13]. Furthermore, the innate and adaptive immune systems of zebrafish have been shown to be remarkably similar to those of other vertebrates, including mammals [14,15]. IPNV is known to infect and replicate in zebra fish remarkably similar to those of other vertebrates, including adaptive immune systems of zebra fish [16,17].

TNFα is a potent pro-inflammatory cytokine that plays important roles in diverse cellular responses, including cell proliferation, differentiation and the induction of other cytokines. TNFα is also a crucial regulator and effector in the immune responses against pathogens by regulating cell death and survival [18]. The production of TNF by cells is initiated by TNF-induced factors and regulated by positive and negative feedback loops. Host cells activate both innate and adaptive responses through TNF-mediated pathways. Multiple virus families encode modulatory proteins to block TNF and TNF-mediated responses [19]. Previous studies have shown that an NF-κB signaling pathway is activated upon TNFRI ligation [20]. The activation of NF-κB transcriptional regulation is

**Table 1**

Primer sequences for duplex siRNA that specifically target the mRNA encoding RT-PCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligo sequence 5' − 3'</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>sense-siRNA</td>
<td>ACCCCCTGCTTGCATATTGCC</td>
<td>NM_001004410</td>
</tr>
<tr>
<td>antisense-siRNA</td>
<td>AGGACCTACAGGAGCAGGGGA</td>
<td>NM_001004410</td>
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</table>

**Table 2**

Primer sequences for duplex siRNA that specifically target the mRNA encoding TNFα and scrambled siRNA.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligo sequence 5' − 3'</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα-siRNA sense-siRNA</td>
<td>TGTGTAGAGGCCGACCAA</td>
<td>NM_001004410</td>
</tr>
<tr>
<td>TNFα-siRNA antisense-siRNA</td>
<td>UACUGTTGCTTCTACTCCTT</td>
<td>NM_001004410</td>
</tr>
<tr>
<td>scrambled-siRNA</td>
<td>TACCCGCTTCTACTCCTT</td>
<td>NM_001004410</td>
</tr>
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**Figure 1**

Western blot analysis showing the expression of TNFα in zebra fish infected with IPNV. (A) Western blot of TNFα in zebra fish infected with IPNV at 24 hpi. (B) Western blot of TNFα in zebra fish infected with IPNV at 48 hpi. The expression of TNFα was detected using anti-TNFα antibody. The blots were normalized to β-actin expression.
Fig. 1. (a) Detection of viral proteins in ZF4 cells after IPNV infection using western blotting. Lanes 1–5 correspond to 0, 3, 6, 12 and 24 h p.i. Each lane was loaded with 30 μg protein from ZF4 cells infected at an MOI of 1. The expression of Actin was used as an internal control. (b) Phase-contrast images of ZF4 cells infected with IPNV (MOI of 1) at 0 (panel A), 6 (panel B), 12 (panel C) and 24 h (panel D) p.i. (c) Fluorescence images of annexin V-fluorescein-labeled and PI-labeled IPNV-infected ZF4 cells (MOI of 1) at 12 and 24 h p.i. Phase-contrast micrographs of IPNV-infected ZF4 cells at 12 h (panel A) and 24 h (panel C) p.i. Fluorescence images of annexin V and PI staining of IPNV-infected cells in panel B (12 h p.i.) and panel D (24 h p.i.) show the apoptotic and necrotic cells.
an immediate-early step of immune responses and anti-apoptosis processes. Viruses also utilize multifunctional viral proteins to disrupt innate responses by targeting specific aspects of the NF-κB-mediated pathway [21]. IPNV may activate NF-κB via tyrosine kinase signaling and regulate apoptosis in virus-infected cells [22]. The activation of NF-κB in IBDV (infectious bursal disease virus)-infected cells was shown to be inhibited by the proteasome inhibitor MG-132, which blocked apoptosis during the early stage of viral replication [23].

In the present work, we conclude that cytokine and metalloproteinase expression is initiated by the TNFα/NF-κB pathway during IPNV infection.

2. Materials and methods

2.1. Cells and viruses

The zebrafish ZF4 cell line, derived from 24-hpf (24 h post-fertilization) zebrafish embryos, was purchased from the American Type Culture Collection (CRL-2050) and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin (Invitrogen, USA). The isolated virus, E1-S, a member of the Ab strain of IPNV, was isolated from Japanese eels in Taiwan. The E1S virus was propagated in ZF4 cell monolayers at a multiplicity of infection (MOI) of 0.01. Infected cultures were incubated at 18°C until extensive cytopathogenic effects (CPE) were observed [24]. Virus plaque assays [25] and TCID50 measurements were performed on confluent monolayers of ZF4 cells.

2.2. Western blot analysis

Approximately 10^5 ZF4 cells/ml were seeded in a 100-mm Petri dish (Nunc, Denmark) and cultivated for more than 24 h. These cells were then infected with IPNV at an MOI of 1 and incubated for 0, 3, 6, 12 or 24 h. At the end of the incubation period, the culture medium was aspirated, and the cells were washed with PBS and lysed in 0.5 ml lysis buffer (10 mM Tris-base, 20% glycerol, 10 mM SDS, 2% β-ME, pH 6.8). Proteins that were present in the cell lysate were separated by SDS-polyacrylamide gel electrophoresis, electroblotted and subjected to immunodetection as described by Kain et al. [26]. The blots were incubated with polyclonal antibodies specific for IPNV VP2 (1:1000 dilution; Microtek, Canada), TNFα (1:1500 dilution; AnaSpec, USA), Mmp9 (1:1500 dilution; AnaSpec), Mmp13 (1:1500 dilution; AnaSpec), NF-κB (1:2000 dilution; AnaSpec), NF-xB (1:2000 dilution; AnaSpec), GATA1 (1:500 dilution; AnaSpec) or actin (1:5000 dilution; AnaSpec) and a 1:50,000 dilution of a peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Sigma Aldrich, USA). Chemiluminescence detection was performed using the Western Exposure Chemiluminescence Kit (GE Healthcare, USA) according to the manufacturer’s instructions. Resulting western blots were scanned with an imaging densitometer (LAS-3000; Fujifilm, Japan), and optical densities of specific proteins were analyzed with Image Gauge software (Fujifilm).

2.3. Apoptotic cells labeled with Annexin V-FITC

At the end of the various incubation times, each sample was removed from the medium and washed with PBS, and the cells...
were incubated with staining solution for 20 min. To determine the differentiation of apoptotic cells, an analysis of phosphatidyl serine (PS) on the outer leaflet of apoptotic cell membranes was performed using an annexin V-fluorescein and propidium iodide (PI) staining kit (Roche, USA) [7]. Apoptotic cells were examined by fluorescence microscopy (Olympus IX70, Japan) using a 488-nm filter for excitation and a 525-nm filter for detection. Necrotic cells were examined by fluorescence microscopy (Olympus IX70) using a 535-nm filter for excitation and a 620-nm filter for detection.

2.4. RNA preparation

Approximately 10^5 ZF4 cells/ml were seeded in a 100-mm Petri dish (Nunc) and cultivated for more than 24 h. These cells were infected with IPNV at an MOI of 1 and incubated for 0, 6, 12 or 24 h. At the end of the incubation period, the culture medium was aspirated, and the cells were washed with PBS and lysed in 1.0 ml TRIzol reagent (Invitrogen, USA). Total RNA was further purified using On-Column RNase-free DNase digestion (QIAGEN, Germany) to remove possible genomic DNA contamination.

2.5. Quantitative real-time PCR

The primers used in quantitative PCR were designed by using Primer Express 2.0 software (Applied Biosystems, USA) and are listed in Table 1. For real-time quantitative PCR, first-strand cDNAs of ZF4 cells were synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Quantitative PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7000 Sequence Detection System (USA). Data are presented as means with standard errors of means (±SE). Quantification of gene expression in IPNV-infected versus uninfected control cells was calculated relative to the ef1a internal gene. Fold changes in gene expression represent mean values derived from three independent experiments.

2.6. Knockdown of TNFα by RNA interference

Duplex small interfering RNAs (siRNA) that specifically targeted TNFα (BC124141) and scrambled siRNA were commercially synthesized (Sigma, Singapore). The sequences of scrambled siRNA and duplex siRNA that specifically targeted TNFα are listed in Table 2. Duplex siRNAs (20 nM) were transfected into ZF4 cells that were cultured in 60-mm-diameter plastic tissue culture plates (Nunc) using the GeneMute siRNA transfection reagent (SignaGen Laboratories, USA). After a 6-h incubation period, 1 ml RPMI 1640 culture medium containing 10% FBS (Invitrogen, USA) was added to each well without removing the transfection reagent. The cells were then infected with IPNV-E1S at an MOI of 1 in 10% FBS RPMI 1640 at 18 °C.

2.7. Treatment with the TNFα-specific inhibitor tyrphostin AG-126

Approximately 10^5 ZF4 cells/ml were seeded in a 60-mm Petri dish (Nunc) and cultured for 24 h. For groups treated with tyrphostin AG-126 (Sigma Aldrich, USA) to inhibit TNFα expression, confluent cells were cultured in 60-mm-diameter plastic tissue culture plates that had been treated with tyrphostin AG-126 for 3 h and then infected with 1 IPNV-E1S at an MOI of in 10% FBS RPMI 1640 (Invitrogen) at 18 °C.

2.8. Treatment with the TNFα inducer lipopolysaccharide

Approximately 10^5 ZF4 cells/ml were seeded in a 60-mm Petri dish (Nunc) and cultured for 24 h. For groups treated with lipopolysaccharide (Sigma Aldrich, USA) to induce TNFα expression, confluent cells were cultured in 60-mm-diameter plastic tissue culture plates that had been treated with 1 μg/ml lipopolysaccharide for 6 h in 10% FBS RPMI 1640 (Invitrogen).

2.9. Treatment with the NF-κB-specific inhibitor panepoxydone

Approximately 10^5 ZF4 cells/ml were seeded in a 60-mm Petri dish (Nunc) and cultured for 24 h. For groups treated with panepoxydone (Alexis, USA) to interrupt the NF-κB signaling pathway, confluent cells were cultured in 60-mm-diameter plastic tissue culture plates that had been treated with 1 μg/ml panepoxydone for 3 h and then infected with IPNV-E1S at an MOI of 1 in 10% FBS RPMI 1640 (Invitrogen) at 18 °C.

2.10. Statistical analyses

All data were analyzed using either a paired or unpaired Student t-tests as appropriate. A p-value of less than 0.05 was considered significant. Errors of mean (±SE) were calculated using the Student t-tests as appropriate. A p-value of less than 0.05 was considered significant. Errors of mean (±SE) were calculated using the Student t-tests as appropriate. A p-value of less than 0.05 was considered significant. Errors of mean (±SE) were calculated using the Student t-tests as appropriate. A p-value of less than 0.05 was considered significant.
a statistically significant difference between the mean values of the groups in all subsequent figures, unless otherwise noted.

3. Results

3.1. Zebrafish embryonic cells infected by IPNV

To determine whether IPNV (MOI = 1) could infect ZF4 cells and induce apoptotic cell death, we first used an anti-IPNV VP2 polyclonal antibody to detect viral protein expression by western blotting. The viral protein VP2 could be detected in IPNV-infected ZF4 cells after 6 h post-infection (p.i.) (Fig. 1a). Fig. 1b shows the morphological changes that occurred in ZF4 cells during virus infection. Whole cells were rounded at 6 h post-infection (Fig. 1b: c) compared to control cells (Fig. 1b: a). These sequential morphological changes were similar to atypical apoptotic morphological changes seen previously in CHSE-214 cells [8]. ZF4 cells were also induced to undergo apoptosis and necrosis by IPNV at 12 and 24 h p.i., as shown by fluorescence images of annexin V-fluorescein and PI staining (Fig. 1c).

3.2. Upregulation of immune response genes in IPNV-infected cells

To demonstrate that innate immune responses were activated during IPNV infection, we examined the transcriptional levels of immune response-related genes. IPNV infection in ZF4 cells resulted in an induction of innate immunity genes, including ifna, ifng, isgf3g and interferon-stimulated genes (mx and isg15) (Fig. 2a). The ifna gene was increased 2-fold at 12 h post-infection. Three interferon-regulated factors, irf1, irf2 and irf4, and their components, irf2bp2, irf2d, irf2q2, irf3p30 and itga5, were also activated following IPNV infection (Fig. 2b and c).

3.3. Upregulation of cytokines and metalloproteinases after IPNV infection in zebrafish cells

To determine whether cytokine expression levels were increased during IPNV infection, the transcription levels of these genes were examined by quantitative RT-PCR after IPNV infection. The gene expression levels of il-1b, il-12, il-15, il-22 and il-26 were increased more than 2-fold at 24 h post-infection. The expression level of the chemokine ccl4 gene was increased more than 8-fold at 12 h and 24 h post-infection. tnfα and tnfβ gene expression levels were increased more than 6-fold after IPNV infection (Fig. 3a). The expression levels of pro-inflammatory molecules il-1, il-12, il-15, il-22, il-26 and ccl4 were increased during IPNV infection. Notably, the gene transcription levels of il-12, ccl4 and tnfα were upregulated beginning at 6 h post-infection. The gene transcriptional levels of nine metalloproteinase members, including mmp1b, mmp9, mmp13, mmp17, mmp20, mmp23, mmp24 and mmp28, were upregulated after IPNV infection, with mmp13 and mmp14 levels increasing more than 8-fold (Fig. 3b).

3.4. Transcriptional regulators were upregulated or downregulated after IPNV infection

The expression levels of transcriptional regulatory genes cebpβ and junb were upregulated from 6 h to 24 h post-infection. The expression of pparγ increased only 3-fold at 6 h post-infection (Fig. 4a). Three immune-response transcription factors, cebpγ, jun and p53, were downregulated from 6 h to 24 h post-infection.
IPNV might suppress the host immune response by repressing the expression of these transcriptional regulators. The expression levels of the NF-κB family members *nfkb*, *arel* and *relc* were upregulated during IPNV infection (Fig. 4c). The expression levels of STAT family genes, including *stat1*, *stat4* and *stat5*, were also upregulated in IPNV-infected cells (Fig. 4d).

3.5. TNFα is effectively suppressed by TNFα-specific siRNA or tyrphostin AG-126

We used the software program Pathway Studio 6.0 (Ariadne Genomic, Inc.) to search for altered genes in a quantitative RT-PCR experiment. TNFα had the greatest downstream influence among
altered immune response-related genes, including other cytokines and transcription factors (S. Fig. 1). We used the inhibitor tyrphostin AG-126 (3-hydroxy-4-nitrobenzylidene malononitrile, C10H5N3O3) to specifically repress the expression of TNFα, as tyrphostin AG-126 inhibits tyrosine kinase activity [27]. The increases in cell death and serum levels of TNFα were shown to be caused by endotoxin when pre-treated with tyrphostin AG-126 in mice [28]. The mRNA and protein expression levels of TNFα were significantly inhibited in virus-infected cells after treatment with tyrphostin AG-126 at 6, 12 and 24 h post-infection (Fig. 5a and b). Tyrphostin AG-126 was concluded to be an inhibitor that suppresses TNFα overexpression. RNA interference was also used to investigate whether knocking down TNFα would affect IPNV pathogenesis. The transcriptional expression of TNFα was reduced to 60.1% and 42.7% by TNFα-specific siRNA #1 and siRNA #2 (20 μM), respectively (Fig. 5c). The protein expression level of TNFα was also significantly decreased following duplex siRNA treatment (Fig. 5d). We used LPS (lipopolysaccharide) as the TNFα inducer in ZF4 cells to determine whether TNFα-specific siRNA or tyrphostin AG-126 could inhibit the expression of TNFα. The RNA and protein expression levels of

![Graphs showing mRNA expression of various genes](Image)

**Fig. 6.** Expression of transcriptional regulators, as detected by quantitative RT-PCR after treatment with TNFα-specific siRNA and tyrphostin AG-126 in IPNV-infected ZF4 cells. (a) *nfkb*, (b) *rel-a*, (f) *cebpb* and (g) *junb*. Quantification of gene expression in IPNV-infected versus uninfected control cells was calculated relative to the *ef1a* internal gene. Bars represent the fold differences in expression levels compared to uninfected cells. Fold changes in gene expression represent mean values derived from three independent experiments. Data shown are the mean ± SD. Student’s *t* tests indicate significant differences compared to untreated control: *, *p* < 0.01. (c) Detection of NF-κB after TNFα-specific siRNA or tyrphostin AG-126 treatment in IPNV-infected ZF4 cells by western blotting. The expression of actin was used as an internal control. (d) Detection of NF-κB after tyrphostin AG-126 treatment in LPS-treated ZF4 cells by western blotting. (e) Detection of NF-κB after tyrphostin AG-126 treatment in LPS-treated ZF4 cells by western blotting.
TNFα induced by LPS were significantly decreased by TNFα-specific siRNA #2 (Fig. 5e and f).

3.6. TNFα is crucial to the activation of NF-κB in IPNV-infected cells

Four transcription factors (NF-κB, Rel-A, C/EBPβ and JUNB) were found to be the downstream effectors of TNFα (S. Fig. 1). We determined whether the expression levels of these three transcriptional regulators were activated through a TNFα-mediated pathway. ZF4 cells were pretreated with TNFα siRNA #2 or tyrphostin AG-126 and then infected with IPNV. The mRNA expression levels of nfkβ and rela were inhibited in TNFα siRNA- or tyrphostin AG-126-treated ZF4 cells following IPNV infection (Fig. 6a and b). The protein expression levels of NF-κB were effectively inhibited in AG-126 or TNFα-specific siRNA-treated cells following IPNV infection (Fig. 6c). The gene expression level of GATA1 was measured as an extra control. We found that the expression of GATA1 did not change in TNFα-specific siRNA or AG-126 pretreated ZF4 cells and then infected by IPNV. We used LPS to induce TNFα over-expression in ZF4 cells that were pre-treated with TNFα-specific siRNA or AG-126. The protein expression levels of NF-κB were also reduced in AG-126 or TNFα siRNA-treated cells following LPS treatment (Fig. 6d and e). The gene expression level of GATA1 was not changed in TNFα-specific siRNA or AG-126 pretreated cells after LPS treatment. The expression levels of the transcriptional regulators cebpβ and junb were not decreased following TNFα inhibition (Fig. 6f and g).

3.7. Cytokine activation occurs through the TNFα/NF-κB pathway

To determine whether cytokines are activated through the TNFα/NF-κB pathway, ZF4 cells were pretreated with TNFα siRNA #2, tyrphostin AG-126 or panepoxydone. Panepoxydone is an NF-κB-specific inhibitor that prevents κB phosphorylation [29]. The transcription levels of ifna (Fig. 7a), ifng (Fig. 7b), mmp9 (Fig. 7c), mmp13 (Fig. 7d), ccl4 (Fig. 7e), mmp14 (Fig. 7f) and il-12 (Fig. 7g) were decreased in panepoxydone-treated ZF4 cells that were infected with IPNV. The transcription levels of ifna (Fig. 7a), ifnr (Fig. 7b), mmp9 (Fig. 7c), mmp13 (Fig. 7d) and ccl4 (Fig. 7e) were decreased in IPNV-infected cells that were treated with siRNA or tyrphostin AG-126. The protein expression levels of Mmp9 and Mmp13 were also decreased in IPNV-infected ZF4 cells after TNFα siRNA or panepoxydone treatment (Fig. 7i). The transcriptional expression level of mmp17 was not decreased after interfering TNFα expression or the NF-κB pathway (Fig. 7h).

4. Discussion

Interferons activate antiviral activities in vertebrate cells. The antiviral activities of recombinant salmon IFNs were demonstrated against IPNV; they also induced Mx protein expression [30]. Zebrafish IFN genes increased Mx gene expression and resistance to infection by snakehead rhabdovirus [31]. Transcripts and protein levels of the teleost fish Mx gene are inducible by type I IFN, poly I:C and virus infection. ISG15 is an ubiquitin-like protein with anti-viral activity and plays an important role in innate immunity. ISG15 protein is highly expressed after stimulating mammalian cells with IFNβ [32]. IPNV could induce ISG15 gene expression and remained high even up to 21 days post-infection in Atlantic cod [33]. In our study, immune-related genes, including ifna, ifnr, mx, isg15 and several interferon regulatory factors, were upregulated following IPNV infection (Fig. 2). IPNV infection might lead to the activation of the innate immune response in zebrafish cells.

IL-1β is a proinflammatory cytokine that is expressed in the early stage of microbial infection [34]. IL-1β also mediates the expression of other cytokines and chemokines through up- or downregulation [35]. Matrix metalloproteinases (MMPs) are enzymes that regulate the cell-matrix composition through their zinc-dependent proteolytic activities. They play an important role in pathological processes, including inflammation, pulmonary diseases, cardiovascular diseases and cancer. IPNV induced the expression of several inflammatory cytokines and mmp family genes involved in immune and inflammatory responses in zebrafish cells (Fig. 3).

TNFα is a potent pro-inflammatory cytokine that is expressed by activated macrophages and lymphocytes. TNFα induces varied responses that can alter apoptotic processes by changing the expression levels of genes involved in immune and early inflammatory responses. Teleost TNF might have diverse effector functions on cytotoxic cells from ectotherms, similar to the effects seen for mammalian TNF [36]. In channel catfish, TNF expression was shown to occur predominantly in macrophages and T cells but not in fibroblasts and B cells [37]. The activation of T cells with TNF induces the expression of TNF receptors and other cytokines [38]. The expression of TNFα was upregulated after treatment with PMA, LPS, recombinant IL-1β and ConA in trout and flounder isolated primary leukocytes [39,40]. Fish TNF was produced in response to the bacterial stimulation of macrophages [41]. TNFα also increases the susceptibility of zebrafish to SVCV and Streptococcus iniae infections [42].

The mRNA level of TNFα was upregulated in cells that were stimulated by pathogens compared to control cells [43,44]. In this study, we found that TNFα expression was increased in IPNV-infected cells at 6, 12 and 24 h post-infection. Multiple virus families encode modulatory proteins to block TNFα and TNFα-mediated responses at multiple levels or modulate key transcription molecules of the TNFα-signaling pathway [19]. TNFα regulates the expression of TLR, costimulatory molecules, MHC class II genes, pro- and anti-inflammatory genes in seabream endocardium endothelial cells (EEC) and macrophages in vitro [42].

TNFα can lead to NF-κB activation or apoptosis depending on the cellular context [19]. NF-κB might play critical roles in the regulation of numerous genes involved in cell proliferation, survival and inflammation in the immediate early pathogen response [45]. Activation of the NF-κB family controls a distinct signaling pathway that leads to caspase 8-dependent apoptosis. Cell death was initiated by NF-κB through the upregulation of anti-apoptotic genes, including c-IAP1, c-IAP2, TRAF1, TRAF2 and C-FLIP [46]. NF-κB activation also induced apoptosis by enhancing pro-apoptotic cytokine expression [45,47]. Previous studies have shown that IPNV infection activates NF-κB via tyrosine kinase signaling, leading to cell death [22]. We found that the expression levels of members of the NF-κB family, including nfkβ, rela and crel, were upregulated after IPNV infection (Fig. 4c).
Previous studies have shown that dsRNA and viral infections are potent activators of NF-κB [48,49]. Birnavirus IBDV uses the p38 MAPK and NF-κB pathways to elicit macrophage activation [50]. A previous study reported that IPNV infection could activate NF-κB in the early replication cycle, apparently by specifically binding to the NF-κB recognition element [22]. The ATP inhibitor genistein inhibited IPNV-induced NF-κB DNA-binding activation, enhanced cell viability and inhibited DNA fragmentation, suggesting that the tyrosine kinase pathway may be involved in IPNV-induced NF-κB activation.

TNFα is the most important pro-inflammatory cytokine during immune responses in fish and mammalian cells. TNFα might regulate the expression levels of cytokines and metalloproteinases through NF-κB in zebrafish cells during viral infection. TNFα might also play a regulatory role in cytokine induction against viral infections in lower vertebrates. Further studies should not only establish zebrafish as a model organism for TNFα/NF-κB signaling in viral pathogenesis but also demonstrate a potential strategy to inhibit viral replication in fishes that are vital to the fishing industry.

Acknowledgements

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.fsi.2011.01.015.

References