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Expression analysis of immune response genes in fish epithelial cells following ranavirus infection

Riikka Holopainen [a,*], Hannele Tapiovaara [a], Jarno Honkanen [b]

[a] Finnish Food Safety Authority Evira, Veterinary Virology Research Unit, Mustialankatu 3, FI-00790 Helsinki, Finland
[b] National Institute for Health and Welfare, Department of Vaccination and Immune Protection, Haartmaninkatu 8, FI-00290, Helsinki, Finland

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ABSTRACT

Ranaviruses (family *Iridoviridae*) are a growing threat to fish and amphibian populations worldwide. The immune response to ranavirus infection has been studied in amphibians, but little is known about the responses elicited in piscine hosts. In this study, the immune response and apoptosis induced by ranaviruses were investigated in fish epithelial cells. Epithelioma papulosum cyprini (EPC) cells were infected with four different viral isolates: epizootic haematopoietic necrosis virus (EHNV), frog virus 3 (FV3), European catfish virus (ECV) and doctor fish virus (DFV). Quantitative real-time PCR (qPCR) assays were developed to measure the mRNA expression of immune response genes during ranavirus infection. The target genes included tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β), β2-microglobulin (β2M), interleukin-10 (IL-10) and transforming growth factor β (TGF-β). All ranaviruses elicited changes in immune gene expression. EHNV and FV3 caused a strong pro-inflammatory response with an increase in the expression of both IL-1β and TNF-α, whereas ECV and DFV evoked transient up-regulation of regulatory cytokine TGF-β. Additionally, all viral isolates induced increased β2M expression as well as apoptosis in the EPC cells. Our results indicate that epithelial cells can serve as an *in vitro* model for studying the mechanisms of immune response in the piscine host in the first stages of ranavirus infection.

1. Introduction

Ranaviruses are large double-stranded DNA viruses of the family *Iridoviridae* that infect fish, amphibians and reptiles [1]. Ranavirus-associated disease outbreaks and mortality have been reported worldwide, and these viruses have become a noticeable threat to both farmed and natural populations of fish and amphibians [2]. Several ranavirus isolates have been characterised in recent decades. Ranaviruses display significant differences in virulence depending on the viral isolate and the species, as well as the age and geographic origin of the host animal [1,3]. In addition to pathogenic isolates, ranaviruses have been isolated from apparently healthy hosts [4–6]. These findings, added to the results from susceptibility studies [6,7], have led to speculations that some host species could act as vectors for ranaviruses. Variation in the host specificity and pathogenicity of different isolates reflects differences not only in virulence, but also in the initiation of the pathogen eradication mechanisms of the host species.

The immune system of bony fish is fundamentally similar to that of mammals and can be divided into innate and adaptive immunity. However, evidence both from fish and mammalian immunology indicates that rather than being two separate entities, innate and adaptive immunity form a multilevel network of different immune mechanisms [8]. Adequate and tightly regulated innate and adaptive immune responses, usually confined to the target organs of virus replication, are vital to host recovery from viral infection. The immunity against and pathogenesis of ranaviruses have been examined using frog virus 3 (FV3), the type species for the genus *Ranavirus*. The essential role of anti-FV3 IgY antibodies [9,10] and $CD8^+$ T cells [11] in adaptive immunity against FV3 has been established using *Xenopus* as an amphibian model. In the innate anti-FV3 immune response in *Xenopus*, Morales et al. [12] reported rapid up-regulation of pro-inflammatory genes such as arginase 1, interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α) as well as active involvement of peritoneal leukocytes. However, immune responses to ranavirus infection in piscine hosts are less well known.

Pro-inflammatory cytokines, such as TNF-α and IL-1β, are involved in the innate immune response, inflammation, apoptosis and cell proliferation [13,14]. The regulation of IL-1β and TNF-α is

* Corresponding author. Tel.: +358 504393974; fax: +358 207724363.
E-mail address: riikka.holopainen@evira.fi (R. Holopainen).

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accomplished by a network of counter-acting cytokines such as transforming growth factor β (TGF-β) and interleukin-10 (IL-10) [15,16]. The MHC class I antigen presentation pathway plays an important role in the immune response against viral infections. MHC class I molecules are heterodimers, consisting of a heavy chain, an integral membrane glycoprotein and β_2-microglobulin (β_2M) protein expressed on the cell surface of all nucleated cells [17]. MHC class I molecules present peptides derived from intracellular proteins, including viral proteins, to cytotoxic T lymphocytes (CTLs), and induce the elimination of infected cells [18].

In vertebrates, epithelial tissues form a barrier between the body and the environment and play an essential role in host defence and regulation of the immune response. In mammals, epithelial cells respond to infection or injury by secreting cytokines and chemokines [19,20]. In addition to acting as primary innate immune effector cells, epithelial cells regulate adaptive immune responses at the level of leukocytes [21].

The aim of this study was to investigate the initiation of the immune response during ranavirus infection in a piscine epithelial cell model. Epithelioma papulosum cyprini (EPC) cells were infected with four different ranaviruses. Quantitative real-time PCR (qPCR) assays were developed to measure changes in the expression of selected immune response genes, including TNF-α, IL-1β, IL-10, TGF-β and β2M. In addition, we investigated the ability of ranaviruses to induce apoptosis.

2. Materials and methods

2.1. EPC cells

EPC cells were selected as a representative cell line to examine the immune response in fish epithelial cells. EPC cells are widely used in diagnostic and research laboratories for viral fish diseases. According to the Manual of Diagnostic Tests for Aquatic Animals by the World Organisation for Animal Health (OIE) [22,23] and results published by Ariel et al. [24], EPC cells are well suited to propagating ranaviruses. The EPC cell line was initially established from proliferative skin lesions of common carp (*Cyprinus carpio*) [25]. However, according to a recent report by Winton et al., the current lineages of EPC cell line have been contaminated and ultimately replaced by fathead minnow (*Pimephales promelas*) epithelial cells [26]. The EPC cells used in this study were kindly provided by Dr E. Ariel (National Veterinary Institute, Denmark). To investigate the origin of the EPC cells, three separate DNA extractions from the cell culture were performed using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The DNA was used to amplify the partial cytochrome oxidase subunit I (COx1) gene of the EPC cells using primers and conditions published by Winton et al. [26]. The amplicons from three separate PCR reactions were used in three independent cloning reactions. The amplicons were inserted into pSC-A-amp/kan vectors and transfected into StrataClone SoloPack competent cells (StrataClone PCR cloning kit, Agilent Technologies, La Jolla, CA, USA). The plasmid DNA was purified from ten colonies from each of the three cloning reactions using QIAprep Spin Miniprep Kit (Qiagen). The inserts in the plasmids were sequenced using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences of all the studied inserts were identical to the published fathead minnow COx1 sequence (GenBank ID EU525089). Our results support the findings of Winton et al. [26], and we conclude that the EPC cells used in this study originated from fathead minnow.

2.2. Viruses

In this study, four ranavirus isolates were used: Epizootic haematopoietic necrosis virus (EHNV), FV3, European catfish virus (ECV) and doctor fish virus (DFV). EHNV was originally isolated from redfin perch (*Perca fluviatilis*) [27], FV3 from northern leopard frog (*Lithobates pipiens*, formerly *Rana pipiens*) [28], ECV from black bullhead (*Ameiurus melas*, formerly *Ictalurus melas*) [29], and DFV from doctor fish (*Labroides dimidatus*) [30]. The isolates were kindly provided by Dr R. Whittington, University of Sydney, Australia (EHNV), Dr W. Ahne, University of Munich, Germany (FV3), Dr G. Bovo, Istituto Zooprofilattico delle Venezie, Italy (ECV), and Dr R. Hedrick, University of California, USA (DFV).

The viruses were propagated in EPC cells at 22 °C according to the OIE Manual [22,23]. Prior to their use in the cell culture experiments, the viruses were titrated in eight replicates of tenfold dilutions, and the titre was determined as the 50% tissue culture infective dose ($TCID_{50}$ ml^{-1}) [31].

2.3. Cell culture experiments

EPC cells were infected with the four ranavirus isolates as described in our previous study [32]. Briefly, about 8.0×10^5 EPC cells per well were grown on 12-well plates (CellBind, Corning, MA, USA) and infected with the isolates with a multiplicity of infection (MOI) of 2.5. The wells were sampled 1, 6, 12, 24, 36, 48 and 72 h after infection by removing the cell culture medium and then scraping the cells into RLT -buffer (RNeasy Mini Kit, Qiagen) for the extraction of total RNA. At each time point, duplicate wells were collected to determine both gene expression levels and viral quantities. The results of the viral quantity measurements have been published earlier [32]. Duplicate negative control wells were collected at each time point.

To obtain cells with a high level of gene expression, EPC cells were grown as described above and incubated with either LPS from *Escherichia coli* 0127:B8 (30 μg ml^{-1}; Sigma–Aldrich, St. Louis, MO, USA), CpG-ODN 1585 (GGTCAACGTTGA; 2 μM; InvivoGen, San Diego, CA, USA) or poly I:C (10 μg ml^{-1}; Sigma–Aldrich). For each stimulant, replicate wells were sampled at 6, 24 and 48 h. The gene expression levels were determined from duplicate wells for each time point. The remaining stimulated cells were pooled and used to generate a standard cDNA. Serial dilutions of standard cDNA were prepared and used to assess the PCR efficiency of the developed qPCR assays. In addition, the standard cDNA template was included as a calibrator for each qPCR assay plate.

2.4. RNA isolation, DNAse treatment and cDNA generation

RNA was isolated from the EPC cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Disruption of the cells was achieved with QIAshredder spin-columns (Qiagen). To ensure the removal of gDNA, on-column DNase I digestion was performed during RNA isolation (RNase-free DNase Set, Qiagen). RNA concentrations were measured with spectrophotometry (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany) and 200 ng of total RNA from each sample was used in cDNA synthesis. cDNA was generated using 2.5 μM random primers (Random Hexamers, Applied Biosystems), 2 μl 10× PCR buffer II (Applied Biosystems), 2 mM dNTPs (Applied Biosystems), 3.75 mM $MgCl_2$, 20 units of RNase Inhibitor (Applied Biosystems) and 100 units of MuLV Reverse Transcriptase (Applied Biosystems). Non-reverse transcriptase controls were included in the cDNA synthesis to monitor possible genomic DNA (gDNA) contamination. The reactions were incubated at 37 °C for 90 min and stored at −20 °C until further use.

Table 1
PCR primers used in the study. R = A/G, W = A/T, Y = C/T, M = A/C, S = C/G.

Target gene	1) 5′-forward primer-3′ 2) 5′-reverse primer-3′	GenBank ID or reference	Amplicon size (bp)
β_2M	1) GCTGTACRTCACTGTACARGGG	L05536	316
	2) TTACATGTTGGGCTCCCAA	NM_001159768	
TNF-α	1) TTACCGCWGGTGATGGTGTC	NM_212859 AJ311800	205
	2) CCTTGGAAGTGACATTTGCTTTT	[33]	
IL-1β	1) CAGCCTGTGTGYYTGGGAAT	AJ245635	166
	2) TGCCGGTYTCYTTCCTGAAG	NM_212844	
IL-10	1) TGGAGTCATCCTTTCTGCTCTG	AB110780	369
	2) CATTTCMSCATATCCCGCTTGAG	AY887900	
TGF-β	1) GGACAGATTCTCAGCAARCTACG	EU086521	362
	2) TACAGCTCCAGTCTCTGCTCTG	AF136947	
40s	1) GAAGATGCAGAGGACCATCG	AB012087	235
	2) GAACTTCTGGAACTGCTTCTTG	BT049673	

2.5. Primer and probe design

At the time of the study, no previously published sequence data from the fathead minnow (EPC cells) were available on the immune genes under investigation. Several PCR primer pairs (Table 1) were designed in order to obtain sequence data from the EPC cells in use. In addition, one reverse primer previously published by Stolte et al. [33] was used in the amplification of partial TNF-α gene. The PCR products were sequenced using the BigDye Terminator v 1.1 cycle sequencing kit (Applied Biosystems) and an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). All sequences, except for IL-1β, were submitted to GenBank; the accession numbers appear in Table 2. The IL-1β sequence was less than 200 bp in length and was therefore unacceptable for the GenBank database. The IL-1β sequence obtained is presented in Fig. 1. The acquired sequences were used to design qPCR assays for mRNA quantitation.

The qPCR primers and probes for β_2M, TNF-α, IL-1β, IL-10, TGF-β and 40s rRNA were designed using the Primer Express 3.0 software provided by Applied Biosystems (Table 2). The primer pairs and probes were selected using the following criteria: primer Tm (58–60 °C), primer length (9–40 nt, optimal 20 nt), probe length (13–30 nt), and amplicon length (50–150 nt). Primers and probes were synthesised by Oligomer Ltd (Helsinki, Finland).

Table 2
qPCR primers and probes used in the study.

Target gene	1) 5′-forward primer-3′ 2) 5′-reverse primer-3′ 3) 5′-(FAM)-probe-(BHQ1)-3′	GenBank ID or reference	Amplicon size (bp)
β_2M	1) CCCTCCTGATATCTCCATTGAACT	JN412132	91
	2) CTGCCAGCCCTTTTCGAA		
	3) TTATCCCTAACTCCCAGCAGACTGACCTGG		
TNF-α	1) CAAGCAATTGGCGAGTGTGT	JN412133	108
	2) CAGTTCCACTTTCCTGATTACTCTGA		
	3) TGCTGCTGTTTGCTTCACGCTCAAC		
IL-1β	1) AGACCAATCTCTACCTCGCTTGTAC	Fig. 1	91
	2) TTAATGGTGTTTAATGTTTCACTGATCTC		
	3) ACGATGCTTCCCCCCACCTGG		
IL-10	1) GATGTCACGTCATGGACGAGAT	JN412134	83
	2) GGACTGGAAGTGGTTCTTCTGTACA		
	3) CTGCGCTTCTACTTGGAAACCATTCTGC		
TGF-β	1) TGTATAACAGCACTGTCGAGCTAAGC	JN412135	83
	2) TCCCTTCTCATTAGGATCTTCTACATC		
	3) AGCAGGCGGCAGATCCTGTACACC		
40s	1) TTTTGAGAAGAGGCATAAGAACATGT	JN412136	78
	2) GTAACGATGTCACCAACAGTCACA		
	3) TGTCCACCTCTCTCCATGCTTCAGGG		

2.6. Optimising PCR conditions and efficiency

All combinations of two concentrations of forward and reverse primers (100 and 300 nM) and probes (100 and 250 nM) for six genes (β_2M, TNF-α, IL-1β, IL-10, TGF-β and 40s rRNA) were used to determine the optimal PCR conditions. The concentrations yielding the lowest cycle threshold (Ct) values in PCR amplification were selected for the qPCR analyses. The PCR specificity for each gene was determined using gel electrophoresis. The PCR efficiency for each target gene was determined using standard curves generated with two-fold dilutions of the standard cDNA template. Inter-assay variability for all assays was determined on at least three separate runs of three replicates of the standard cDNA template. Based on the results, the mean Ct values, standard error of the mean (SEM) and coefficient of variation (CV) were calculated.

2.7. Quantitative real-time PCR

All qPCR assays were carried out in the StepOnePlus Real-Time PCR system (Applied Biosystems). The reactions contained 5 μl TaqMan Universal PCR Master Mix with AMPerase UNG (Applied Biosystems), 300 nM forward and reverse primer, 100 nM probe, 3 μl of cDNA and water up to a final volume of 10 μl. All samples were run in triplicate. The standard cDNA template was included in each plate in order to monitor the reproducibility of the qPCR assays.

The cycling conditions were: initial incubation steps at 50 °C for 2 min (AmpErase UNG activation), and at 95 °C for 10 min (hot-start DNA polymerase activation), followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.8. Apoptosis

The ability of the four ranaviruses to induce apoptosis in EPC cells was examined. Approximately 4.0×10^5 EPC cells were plated onto glass cover slips and infected with EHNV, FV3, ECV or DFV with a MOI of 2.5. At 6, 24 and 48 h after infection, three separate staining reactions for each viral isolate were performed. The cells were stained with 5 μg/ml Hoechst 33258 (HO, Sigma–Aldrich) and 5 μg/ml propidium iodide (PI, Sigma–Aldrich) for 30 min at 37 °C. Negative control cells were included in each staining. After staining, the cells were rinsed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and rinsed three times with PBS. The cover slips were mounted on the slides, and viewed with a Leica DM 400B (Wetzlar, Germany) fluorescence microscope. The proportion of apoptotic cells in each cover slip was estimated by counting the cells from 10 randomly chosen areas. Based on the results, mean values and SEM were calculated for each viral treatment group and time point.

Both HO and PI bind to DNA. HO passes freely through all cell membranes and stains DNA blue. PI enters necrotic and late-phase apoptotic cells and induces red staining of DNA. Apoptotic cells can be identified by their fragmented nuclei with blue staining (HO). Necrotic cells are stained with both HO and PI, and exhibit white fluorescence.

3. Data analysis

The raw qPCR data were analysed with StepOne Software v2.0 (Applied Biosystems). The Ct values were determined using a constant threshold value 0.03 for all genes and samples. The relative quantitation of β_2M, TNF-α, IL-1β, IL-10 and TGF-β expression was calculated using the comparative Ct method [34]. The

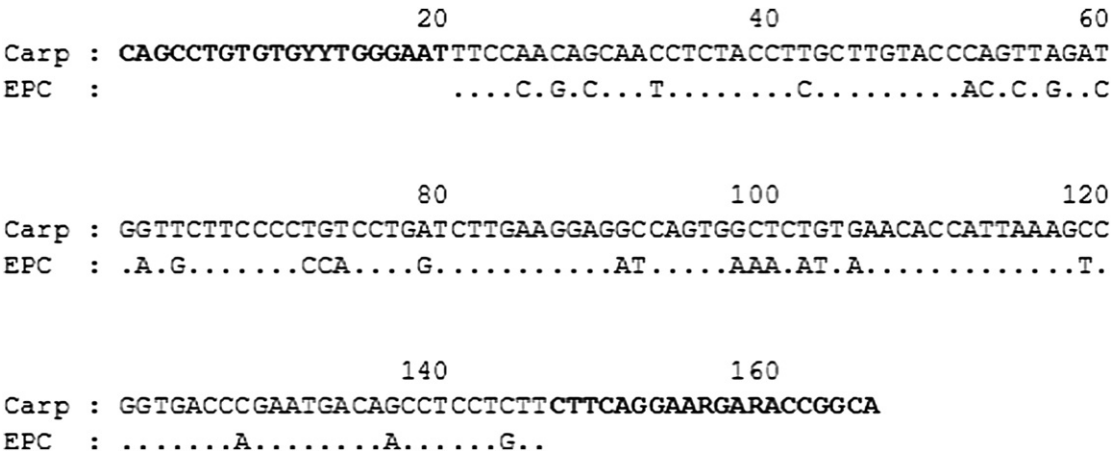

Fig. 1. Nucleotide alignment of partial IL-1β mRNA sequences of carp (GenBank ID: AJ245635) and EPC cells (fathead minnow). Primer sequences appear in bold.

relative quantitation value of the target, normalised to an endogenous control and relative to the calibrator, is expressed as $2^{-\Delta\Delta Ct}$. In this study, ΔCt = Ct of the target gene (β_2M, TNF-α, IL-1β, IL-10 or TGF-β) − Ct of the endogenous control gene (40s). ΔΔCt = ΔCt of the sample − ΔCt of the calibrator sample. The fold change in the expression values was calculated by dividing the relative gene expression value of the treated sample by the relative gene expression value of the negative control.

To compare differences between the treatment groups and the negative control group, statistical analyses were performed with one-way analysis of variance (ANOVA) and Dunnett's test. If the homoscedasticity criteria of the data were unmet, non-parametric Kruskall–Wallis and Dunn's tests were used. The *p*-values were adjusted for multiple comparisons.

The viral quantities (virions/host cell) of the samples studied here have been determined and published previously [32]. In this study, the viral quantities were reanalysed to obtain a relative viral load value by dividing the viral quantity for each viral isolate and time point by the initial infective dose (MOI 2.5). The relative values obtained were used to test the dependence of the relative immune gene expression and the relative viral load with Spearman's rank correlation. All analyses were performed using Graph Pad Prism 5 software (Graph Pad Software Inc., La Jolla, CA, USA) and the significance level was 5%.

4. Results

4.1. Gene sequences of the EPC cells

Due to the lack of published fathead minnow immune gene sequences, partial β_2M, TNF-α, IL-1β, IL-10, TGF-β and 40s rRNA genes of EPC cells were sequenced (Table 2, Fig. 1) in order to design primers and probes for mRNA expression measurements. The obtained nucleotide (nt) and amino acid (aa) sequences of the genes studied were aligned and analysed with published sequences of common carp. The differences between the gene sequences of EPC cells (fathead minnow) and carp were the following: 48 nt and 13 aa differences in β_2M, 26 nt and 11 aa differences in TNF-α, 28 nt and 10 aa differences in IL-1β, 39 nt and 9 aa differences in IL-10, 69 nt and 29 aa differences in TGF-β and 9 nt and 1 aa differences in 40s rRNA. The GenBank IDs of the carp sequences used in the analyses were L05536 for β_2M, AJ311800 for TNF-α, AJ245635 for IL-1β, AB110780 for IL-10, AF136947 for TGF-β and AB012087 for 40s rRNA.

4.2. Assay optimisation

Of the combinations of primer and probe concentrations evaluated, 300 nM of the forward and reverse primer and 100 nM of the probe used to target the six representative genes produced the best amplification result. Each primer pair generated a single amplification product; the specificity was confirmed with gel electrophoresis (results not shown). No fluorescence signal above the threshold was detected from the non-reverse transcriptase controls in PCR amplification, indicating that the cDNA contained no gDNA contamination.

4.3. PCR efficiency and inter-assay variability

The PCR efficiency for each gene was determined from the slope of the standard curve generated with two-fold dilutions of the standard cDNA template prepared from RNA drawn from EPC cells stimulated with LPS, CpG-ODN and poly I:C. Efficiency (*E*) was calculated from the slope values using StepOne Software v2.0 (Applied Biosystems) and the equation $E = 10^{(-1/slope)} - 1$. All six PCR assays produced a strong linear fit with the cDNA template dilutions ($R^2 \geq 0.95$, Table 3) and the PCR efficiency was ≥92% for all assays.

Inter-assay variability was measured using the standard cDNA template. For every qPCR assay, 40s was measured from this pooled sample. In addition, the target gene in question was measured from the standard cDNA template in each qPCR run. The inter-assay CVs of the mean Ct values were ≤2.6 (%) for all genes

Table 3
Standard curves, inter-assay variability and regression analyses of the developed qPCR assays. R^2: coefficient of determination, *E*: PCR efficiency, Ct: cycle threshold value, SEM: standard error of the mean, CV: coefficient of variation, ΔCt: Ct (target) − Ct (40s).

	Standard curve (Ct)			Inter-assay variability			Regression analysis (ΔCt)	
Gene	Mean slope	R^2	*E* (%)	Mean Ct	SEM	CV (%)	Mean slope	R^2
β_2M	−3.19	0.99	105	20.2	0.18	2.6	−0.001	0.45
TNF-α	−3.34	0.99	99	28.3	0.09	0.9	−0.001	0.19
IL-1β	−3.53	0.98	92	29.2	0.08	0.8	0.000	0.00
IL-10	−3.33	0.95	99	30.9	0.06	0.5	−0.002	0.46
TGF-β	−3.17	0.99	106	24.2	0.04	0.5	−0.001	0.67
40s	−3.47	0.99	95	18.9	0.04	1.4	–	–

(Table 3), indicating that all the qPCR assays developed were highly reproducible.

ΔCt values for each target gene were calculated using the Ct values of the target and the reference gene (40s) obtained from the standard curve. Regression analysis was performed using the ΔCt values and the respective concentrations of the dilution series of the standard cDNA template. The slope of the regression curve for each target gene was approximately 0, indicating that the comparative Ct method could be used in relative quantitation with the developed qPCR assays.

4.4. Gene expression in the ranavirus-infected and immunostimulated EPC cells

Ranavirus infection induced changes in the expression of all immune genes studied. A statistically significant increase in β2M expression compared to the negative control was first detected at 6 h post infection (p.i.) in cells infected with FV3, ECV and DFV (Fig. 2). At 12 h p.i., a higher than two-fold expression change was detected in all viral treatment groups and the greatest increase was detected at 36 h p.i. in cells infected with ECV (4.2-fold, Table 4). In EHNV-infected cells, a statistically significant increase in the β2M expression was only detected at 72 h p.i. TNF-α expression was lower in all viral treatments than in the negative control at 1 h p.i. At subsequent time points, the expression levels rose and statistically significant changes were measured in all viral treatment groups. The greatest fold change was detected at 12 h p.i. in EHNV-infected cells (16.4-fold). Compared to β2M and TNF-α, IL-1β expression increased more slowly in the ranavirus-infected cells. At 36 h p.i., a more than two-fold increase was detected for all viral treatments. Of all the viral isolates, FV3 induced the most notable increase: 61.6-fold at 72 h p.i. In EHNV-infected cells, the greatest fold change was detected at 36 h p.i. (11.8-fold). In ECV- and DFV-infected cells, changes in the IL-1β expression were not statistically significant at any of the time points studied, even though a greater than three-fold increase was detected in both groups. IL-10 expression levels were elevated at 1 h p.i. in FV3-, EHNV- and DFV-infected cells. At later time points, the expression fold change values decreased and by the end of the experiment, IL-10 expression was lower in all viral treatments than in the negative control ($p < 0.001$). TGF-β expression was lower in the virally infected cells than in the negative controls at the first time points. In DFV-infected cells, a modest but statistically significant increase in gene expression was detected at both 6 and 12 h p.i. (2.5 and 1.9-fold, respectively). Likewise, in ECV-infected cells, a statistically significant 2.2-fold increase was detected at 36 and 72 h p.i. FV3 and EHNV induced negligible changes in TGF-β expression levels during the study period. LPS induced a statistically significant increase in both IL-1β and TNF-α expression. In cells treated with CpG-ODN, a greater than two-fold increase compared to the negative control was detected in β2M, IL-1β and TNF-α expression. Poly I:C induced a statistically significant increase in β2M, TNF-α, IL-10 and TGF-β expression, and a more than two-fold increase in IL-1β expression.

4.5. Relative immune gene expression and viral load

The correlation between immune gene expression and relative viral load was examined. The viral load for each ranavirus was expressed as the viral quantity relative to the initial infective dose (MOI 2.5) (Fig. 3). The relative viral load correlated significantly with IL-1β expression in FV3-, EHNV- and DFV-infected cells (Fig. 4, $p < 0.05$). IL-10 expression correlated with the relative viral load of only one ranavirus, DFV ($p = 0.034$). The expression levels of the other immune genes studied showed no correlation with the viral loads.

4.6. Apoptosis

All four ranaviruses studied induced chromatic condensation, the characteristic phenotype associated with apoptosis, in EPC cells. No apoptotic changes were observed in the negative controls cells. As Fig. 5 illustrates, negative control cells contained uniformly stained nuclei, whereas condensed chromatin and multiple apoptotic bodies were visible in ranavirus-infected cells. With all ranavirus isolates, the proportion of apoptotic cells observed was relatively low, less than 5%, at the three time points studied (Table 5). The highest percentage of apoptotic cells (mean value 4.56%) was detected at 48 h p.i. in cells infected with FV3 in comparison to other viral treatments ($p < 0.001$). Necrotic cells were rare in all viral treatment groups.

5. Discussion

In this study, we developed qPCR assays to measure and characterise the expression of selected immune genes in EPC cells infected with ranaviruses. Based on viral gene sequences, FV3, EHNV and ECV are more closely related to each other than to DFV [35,36]. Despite their genetic dissimilarity, all four isolates induced an immune response in fish epithelial cells. However, some differences in the expression levels of the immune genes were detected between the viral treatment groups.

Changes in the gene expression of pro-inflammatory cytokines were induced by all four ranaviruses. The up-regulation of both TNF-α and IL-1β was most notable in cells infected with FV3 and EHNV, whereas ECV and DFV only induced statistically significant fold change in TNF-α expression. In the pro-inflammatory signalling cascade, TNF-α is usually the first cytokine to be secreted, which leads to the downstream expression of IL-1β and chemokines such as IL-8 [37]. IL-1β expression correlated strongly with the relative viral loads of FV3, EHNV and DFV, indicating that the growing number of virions enhanced pro-inflammatory cytokine production in EPC cells. Similar up-regulation of TNF-α and IL-1β has been reported with FV3-infected *Xenopus* [12]. Another ranavirus, Singapore grouper iridovirus, reportedly induced an increase in the TNF-α expression in grouper (*Epinephelus* spp.) spleen cells [38]. In addition to their various roles in the innate immune response, TNF-α and IL-1β have been associated with the induction of apoptosis. The binding of TNF-α to a specific cell surface receptor, TNFR1, initiates the signalling pathway leading to apoptosis [39]. Furthermore, both TNF-α and IL-1β stimulate the production of another antiviral cytokine, IFN-γ, which is a potent mediator of apoptosis [40,41]. The elevation detected in TNF-α and IL-1β expression appeared to be in accordance with the apoptotic changes observed in this study. The initiation of apoptosis suggests that protein products of the immune response genes under study were actually produced in EPC cells. In previous studies, FV3 has induced apoptosis in piscine, mammalian, and amphibian cells [12,42].

β2M is an essential part of the structure, correct folding and cell surface expression of MHC class I molecules [17]. Here, β2M expression increased relatively rapidly after viral induction in all four ranavirus treatment groups. The increased β2M expression in EPC cells suggests that in a piscine host ranaviruses induce an antigen-specific immune response leading to the activation of cytotoxic lymphocytes. Other viruses infecting fish have been reported to induce increased β2M expression. In rainbow trout (*Oncorhynchus mykiss*) infected with infectious haematopoietic necrosis virus (IHNV), β2M transcription was

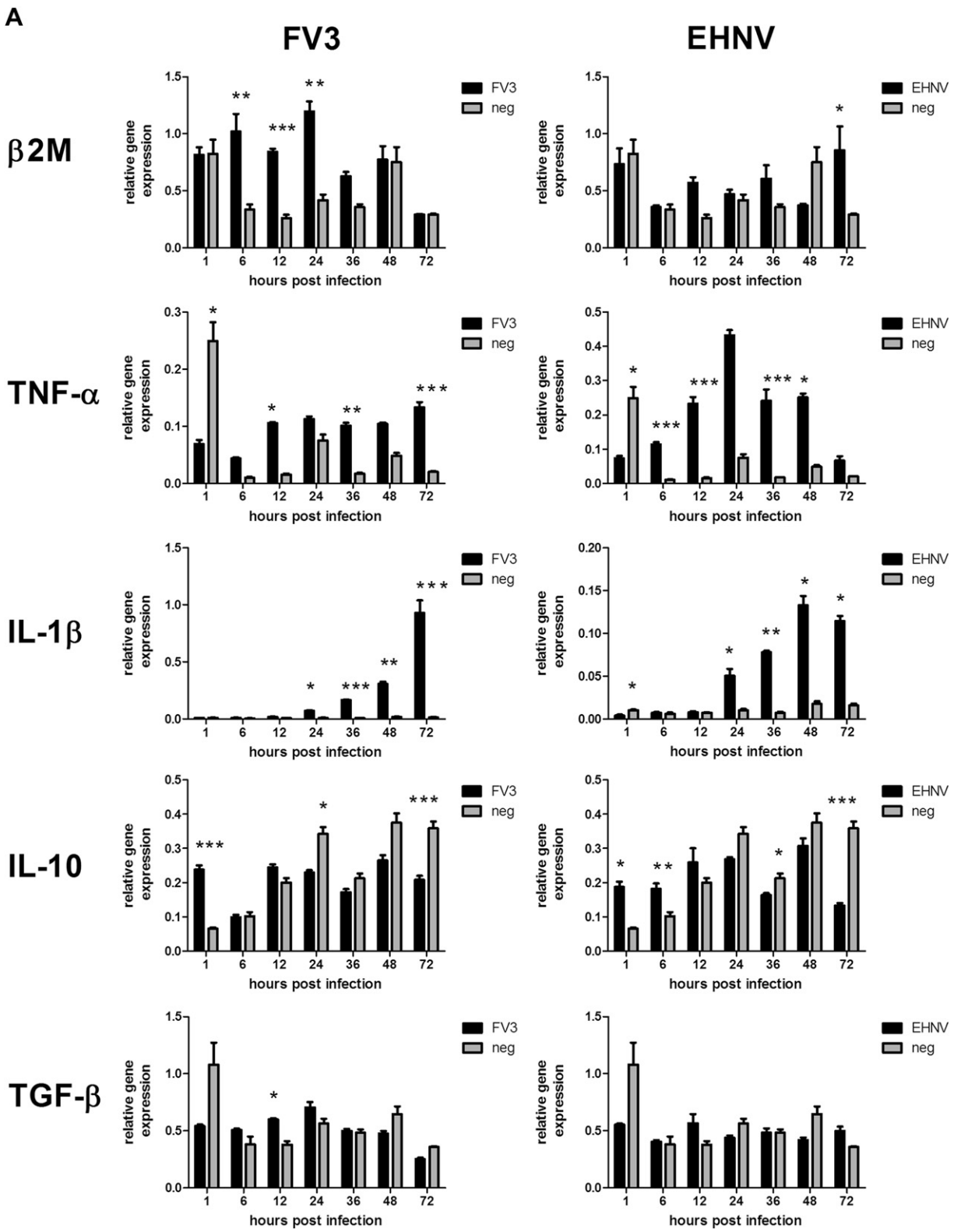

Fig. 2. Relative gene expression of five immune genes in four ranavirus treatment groups and in the negative control: (A) FV3 and EHNV, (B) ECV and DFV. Error bars represent standard error of the mean. Statistically significant differences between the viral treatment groups and negative control are marked with asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

enhanced in the spleen and intestine [43]. Additionally, β2M expression was increased in the head kidney of Atlantic salmon (*Salmo salar*) infected with infectious salmon anaemia virus (ISAV) [44].

Limiting the immune response is as crucial for the host as protection against the infecting pathogen [45]. IL-10 regulates inflammatory responses and potently inhibits the production of multiple cytokines, including IL-1β and TNF-α [15]. In fish, the

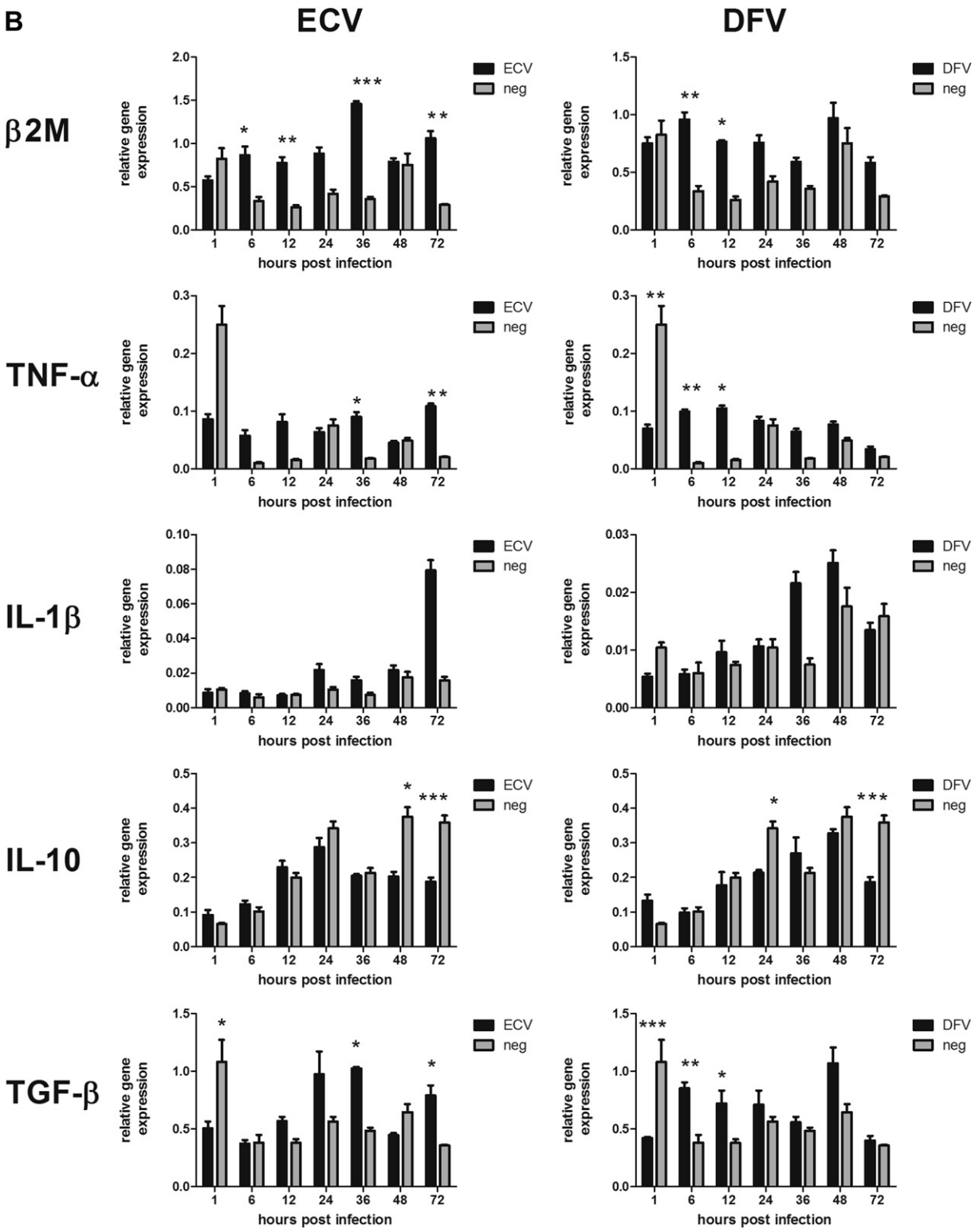

Fig. 2. (*continued*).

regulatory effect of IL-10 on IL-1β expression has been observed in Indian major carp (*Catla catla*) [46]. In the present study, IL-10 was up-regulated only at the early time points in the ranavirus-infected EPC cells. By the end of the study period, IL-10 expression was lower in all ranavirus treatment groups than in the negative control. The observed down-regulation of IL-10 may be associated with the increased expression of TNF-α and IL-1β in the virally infected cells. Even though IL-10 is mainly produced by the cells of the immune system [47], human epithelial cells have been reported to regulate immune response through the expression of IL-10 [48,49]. Our observation that EPC cells are able to express IL-10 and that the expression levels seem to respond to ranavirus

Table 4
Mean fold change in gene expression ± standard error in seven treatment groups for five different genes. The value for the negative control group for each gene and treatment is always 1, and is therefore not shown. Greater than two-fold increases or decreases in gene expression relative to the negative control appear in bold, and statistically significant changes are marked with asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. ND = not done.

Gene	Treatment	1 h	6 h	12 h	24 h	36 h	48 h	72 h
β2M	FV3	1.0 ± 0.1	**3.2 ± 0.5****	**3.6 ± 0.6*****	**3.2 ± 0.7****	1.8 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
	EHNV	0.9 ± 0.0	1.2 ± 0.2	**2.3 ± 0.3**	1.3 ± 0.3	1.8 ± 0.4	0.6 ± 0.1	**3.0 ± 0.8***
	ECV	0.7 ± 0.1	**2.7 ± 0.3***	**3.3 ± 0.7****	**2.3 ± 0.4**	**4.2 ± 0.3*****	1.2 ± 0.2	**3.7 ± 0.4****
	DFV	1.0 ± 0.2	**3.1 ± 0.5****	**3.2 ± 0.5***	**2.0 ± 0.4**	1.7 ± 0.2	1.3 ± 0.1	**2.0 ± 0.2**
	LPS	ND	1.4 ± 0.2	ND	1.2 ± 0.2	ND	1.8 ± 0.1	ND
	CpG-ODN	ND	1.2 ± 0.2	ND	**2.5 ± 0.3***	ND	0.7 ± 0.2	ND
	Poly I:C	ND	**5.3 ± 1.8****	ND	0.8 ± 0.1	ND	**2.4 ± 0.2**	ND
TNF-α	FV3	**0.3 ± 0.0***	**5.0 ± 0.7**	**7.4 ± 0.9***	1.6 ± 0.2	**6.1 ± 0.7****	**2.2 ± 0.2**	**6.5 ± 0.4*****
	EHNV	**0.3 ± 0.0***	**12.7 ± 1.7*****	**16.4 ± 2.8*****	**6.3 ± 0.8**	**15.0 ± 3.2*****	**5.5 ± 0.6***	**3.3 ± 0.6**
	ECV	**0.4 ± 0.0**	**6.1 ± 1.0**	**5.9 ± 1.3**	0.9 ± 0.1	**5.3 ± 0.6***	1.0 ± 0.1	**5.3 ± 0.2****
	DFV	**0.3 ± 0.1****	**11.4 ± 1.8****	**7.3 ± 0.9***	1.2 ± 0.1	**3.7 ± 0.2**	1.6 ± 0.1	1.7 ± 0.3
	LPS	ND	**362 ± 77.3*****	ND	**22.4 ± 5.3***	ND	**138.4 ± 18.1*****	ND
	CpG-ODN	ND	**2.7 ± 0.3**	ND	**0.3 ± 0.0**	ND	1.8 ± 0.2	ND
	Poly I:C	ND	**9.4 ± 2.4***	ND	**0.3 ± 0.1**	ND	**7.2 ± 1.8**	ND
IL-1β	FV3	0.9 ± 0.2	**2.5 ± 0.9**	**2.2 ± 0.6**	**7.8 ± 1.6***	**25.4 ± 4.5*****	**20.9 ± 3.8****	**61.6 ± 8.8*****
	EHNV	**0.4 ± 0.1***	1.8 ± 0.6	1.1 ± 0.2	**5.0 ± 0.6***	**11.8 ± 1.9****	**9.2 ± 2.0***	**7.9 ± 1.1***
	ECV	0.8 ± 0.2	**2.1 ± 0.8**	1.0 ± 0.1	**2.3 ± 0.4**	**2.5 ± 0.6**	1.7 ± 0.5	**5.4 ± 0.7**
	DFV	**0.5 ± 0.1**	1.6 ± 0.6	1.3 ± 0.2	1.2 ± 0.3	**3.2 ± 0.4**	1.8 ± 0.5	0.9 ± 0.2
	LPS	ND	**5.9 ± 1.6***	ND	**3.0 ± 0.4**	ND	**6.4 ± 1.4**	ND
	CpG-ODN	ND	0.9 ± 0.2	ND	0.6 ± 0.1	ND	**2.5 ± 0.4**	ND
	Poly I:C	ND	**2.3 ± 1.1**	ND	1.0 ± 0.2	ND	**2.5 ± 0.9**	ND
IL-10	FV3	**3.6 ± 0.2*****	1.0 ± 0.2	1.3 ± 0.1	0.7 ± 0.1*	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.0***
	EHNV	**2.9 ± 0.3***	**2.0 ± 0.3****	1.3 ± 0.2	0.8 ± 0.0	0.8 ± 0.0*	0.9 ± 0.1	**0.4 ± 0.0*****
	ECV	1.4 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 0.1*	**0.5 ± 0.1*****
	DFV	**2.0 ± 0.3**	1.0 ± 0.1	0.9 ± 0.2	0.6 ± 0.0*	1.3 ± 0.2	0.9 ± 0.1	**0.5 ± 0.1*****
	LPS	ND	1.6 ± 0.2	ND	0.6 ± 0.1	ND	1.4 ± 0.1	ND
	CpG-ODN	ND	0.9 ± 0.2	ND	0.7 ± 0.1	ND	1.0 ± 0.2	ND
	Poly I:C	ND	**3.1 ± 0.3*****	ND	1.4 ± 0.2	ND	**2.1 ± 0.1**	ND
TGF-β	FV3	0.6 ± 0.1	1.5 ± 0.3	1.6 ± 0.1*	1.3 ± 0.2	1.0 ± 0.1	0.8 ± 0.0	0.7 ± 0.0
	EHNV	0.6 ± 0.1	1.2 ± 0.2	1.5 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	1.4 ± 0.1
	ECV	**0.5 ± 0.0***	1.1 ± 0.2	1.5 ± 0.1	1.7 ± 0.3	**2.2 ± 0.1***	0.7 ± 0.1	**2.2 ± 0.2***
	DFV	**0.5 ± 0.1*****	**2.5 ± 0.3****	**1.9 ± 0.3***	1.3 ± 0.3	1.2 ± 0.1	1.6 ± 0.1	1.1 ± 0.1
	LPS	ND	1.5 ± 0.2	ND	1.1 ± 0.1	ND	1.9 ± 0.1	ND
	CpG-ODN	ND	1.2 ± 0.2	ND	1.6 ± 0.2	ND	0.9 ± 0.1	ND
	Poly I:C	ND	**3.2 ± 0.5****	ND	0.9 ± 0.1	ND	**2.3 ± 0.1**	ND

infection may provide an interesting insight to the immune regulatory functions of fish epithelial cells.

TGF-β is a pleiotropic cytokine that controls the initiation and resolution of inflammatory responses [16]. Parasitic pathogens reportedly induced the up-regulation of TGF-β expression in rainbow trout [50,51]. Infection with IHNV, on the other hand, caused no significant changes in TGF-β expression in rainbow trout [52]. In this study, ECV and DFV transiently induced TGF-β expression. Even though the magnitude of the fold change in expression was relatively modest, it was statistically significant at two time points for both isolates. In humans, TGF-β has been shown to enhance viral replication [53,54]. The slight increase in TGF-β expression in the EPC cells infected with ECV and DFV could be related to the replication strategy of these viral isolates.

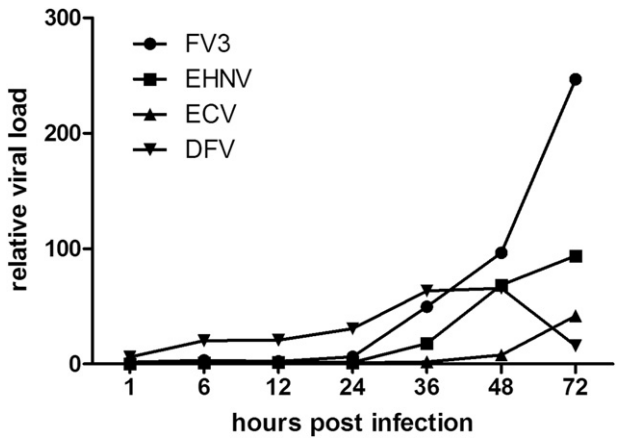

Fig. 3. Relative viral load for four ranavirus isolates and seven time points studied. Relative viral load units are expressed as the ratio of absolute viral quantity to the initial infective dose (MOI 2.5).

In addition to the ranavirus treatments, EPC cells responded to the other microbial stimulants. As in the results reported from carp and rainbow trout head kidney phagocytes [33,55,56], the bacterial mimic LPS induced a notable increase in both TNF-α and IL-1β expression. Additionally, the modest increase in β2M, TNF-α and IL-1β expression induced by bacterial DNA (CpG-ODN) corresponds to the previous findings in mammals and rainbow trout [57–59]. Poly I:C, a synthetic viral-like dsRNA, appeared to be a strong stimulant for EPC cells: greater than two-fold increase was detected in all immune genes studied. Similar results have been reported in previous studies, in which up-regulation of β2M and TGF-β expression was observed upon poly I:C stimulation in fish [52,60,61].

The species of host animal is known to affect the pathogenicity of ranaviruses [1,2]. EHNV and ECV are pathogenic to certain fish species [6,7,27,29,62], whereas FV3, a known amphibian pathogen, has been shown to persist in fish without causing mortality [6,7]. Our results provide preliminary evidence that EPC cells produce a distinct immune response pattern against different ranavirus isolates. It is clear that more studies with primary cells and ultimately with living piscine hosts are needed to confirm our results and further elucidate the immune processes activated

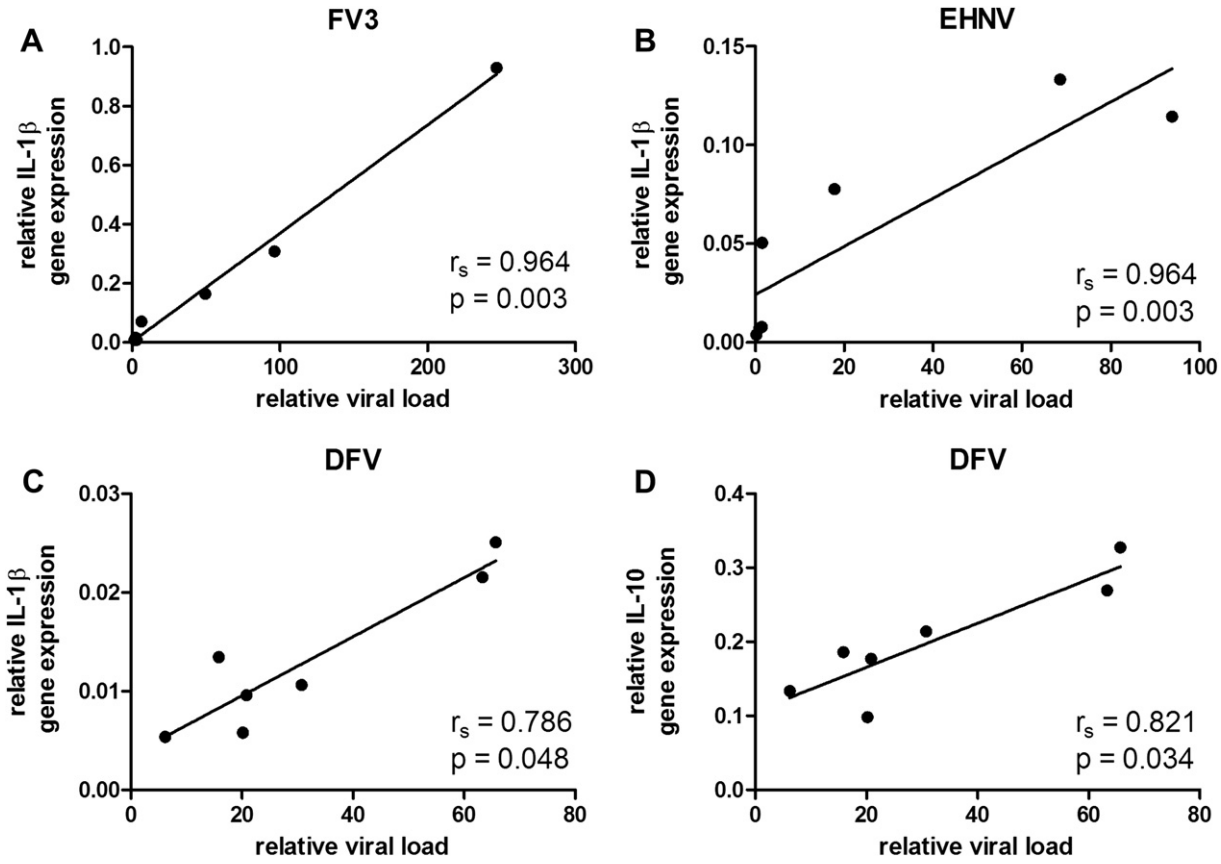

Fig. 4. Association between relative IL-1β (A–C) and IL-10 (D) expression and relative viral load. r_s: Spearman's rank correlation coefficient.

by different ranaviruses in different host species. In addition, since the products of the immune genes investigated here may undergo significant post-transcriptional and post-translational modifications, it would be important to focus on the detection of the protein products in future studies.

In conclusion, the results of this study indicate that ranaviruses elicit the expression of TNF-α, IL-1β and TGF-β, catalyse the MHC class I pathway and induce apoptosis in fish epithelial cells. The methods described here allow further studies on the mechanisms of host immune response to ranavirus infection.

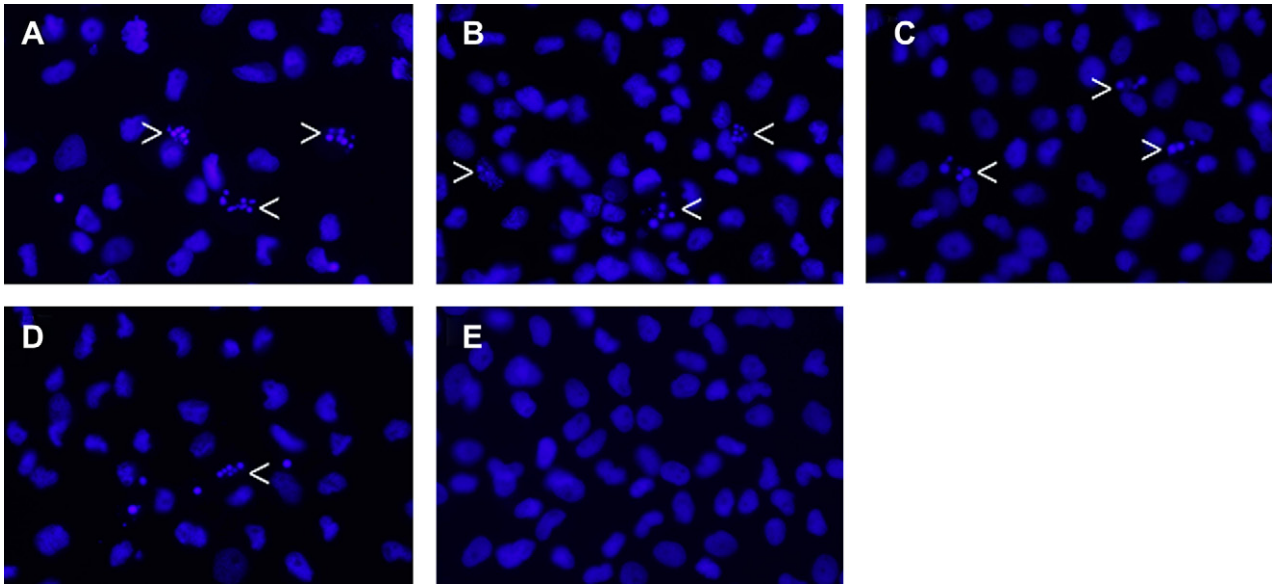

Fig. 5. Induction of apoptosis in EPC cells infected with (A) FV3, (B) EHNV, (C) ECV and (D) DFV. (E) Negative control cells. Apoptotic bodies are indicated with arrowheads. Pictures were taken at 400× magnification.

Table 5
The percentage of apoptotic EPC cells (mean ± standard error) calculated from three different time points in four ranavirus treatment groups and a negative control.

Time p.i.	FV3	EHNV	ECV	DFV	Neg
6 h	2.17 ± 0.21	1.25 ± 0.11	1.04 ± 0.08	2.65 ± 0.66	0.0 ± 0.0
24 h	4.19 ± 0.28	2.42 ± 0.22	1.84 ± 0.16	2.49 ± 0.26	0.0 ± 0.0
48 h	4.56 ± 0.24	2.52 ± 0.15	1.19 ± 0.09	2.31 ± 0.19	0.0 ± 0.0

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