Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma

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ABSTRACT

Interferon-gamma has been typically described as a pro-inflammatory cytokine playing an important role in the resolution of both viral and bacterial diseases. Nevertheless, some anti-inflammatory functions are also attributed to this molecule. In this work we have characterized for the first time the turbot (*Scophthalmus maximus*) *interferon-gamma* gene (*ifng*) and its expression pattern under basal conditions, after type I IFNs administration, and viral and bacterial infection. The intramuscular injection of an expression plasmid encoding turbot Ifng (pMCV1.4-*ifng*) was not able to affect the transcription of numerous immune genes directly related to the activity of IFN-gamma, with the exception of *macrophage-colony stimulating factor* (*csf1*). It was also unable to reduce the mortality caused by a Viral Hemorrhagic Septicemia Virus (VHSV) or *Aeromonas salmonicida* challenge. Interestingly, at 24 hours post-infection, turbot previously inoculated with pMCV1.4-*ifng* and infected with VHSV showed an increase in the expression of pro-inflammatory cytokines and type I IFNs compared to those fish not receiving expression plasmid, indicating a synergic effect of Ifng and VHSV. On the other hand, some macrophage markers, such as the *macrophage receptor with collagenous structure* (*marco*), were down-regulated by Ifng during the viral infection. Ifng had the opposite effect in those turbot infected with the bacteria, showing a reduction in the transcription of pro-inflammatory and type I IFNs genes, and an increase in the expression of genes related to the activity of macrophages.

Keywords: interferon-gamma, turbot, teleost, virus, bacteria, inflammation, type I interferons, macrophages activity

1. Introduction

Interferons (IFNs) are a family of multifunctional cytokines with central importance against viral infections, among other immune functions [1]. These proteins are produced when a pathogen is detected by the pattern recognition receptors (PRRs), which results in the activation of different signaling pathways [2]. In fish, two subfamilies of IFNs were established in basis to differential structural and functional properties, type I and type II IFNs [3]. Type I IFN subfamily comprises a group of typical antiviral proteins, and a variable number of type I IFNs were described in several teleost species [3]. Indeed, it has been shown that type I IFNs from the same teleost can possess distinct properties and capabilities, suggesting in some cases complementary or specialized roles, as was previously observed in turbot [4]. In contrast, IFN-gamma (type II IFN) is a markedly different IFN, possessing some ability to interfere with viral infections but being mainly an immunomodulatory molecule [5,6]. This cytokine is produced by diverse immune-related cell types, although T and NK cells are the major sources, and it is implicated in several aspects of the immunity, such as activation of macrophages, stimulation of antigen presentation, orchestration of leukocyte-endothelium interactions, and effects on cell proliferation and apoptosis, among others [7]. Regarding inflammation, IFN-gamma was typically described as a pro-inflammatory protein, although this appreciation seems to not be totally absolute because, in some cases, protective anti-inflammatory functions were related with this cytokine [8,9].

Unlike mammals, it has been shown that some bony fish, especially cyprinids, possess two type II interferon genes [10-14]. A teleost-specific duplication of the *ifng* gene originated the additional IFN-gamma related (*ifngrel*) gene, although it is not a clear homologue of mammalian IFN-gamma [3]. The inflammatory functions of teleost type II IFNs have not been fully characterized, especially in the case of those species possessing two genes. Some studies revealed that Ifng has the ability to induce the expression of pro-inflammatory cytokines [15-17]. Nevertheless, it was reported that zebrafish Ifng lacks the powerful pro-inflammatory activity of its mammalian counterpart, although it helped to potentiate the induction of antiviral and pro-inflammatory genes by type I IFNs [18]. It was observed that, as in mammals, fish Ifng induces the activation of phagocytic cells by increasing the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), the enhancement of phagocytosis

and the up-regulation in the expression of different immune genes in this cell type [13,16,17,19].

Exploring the turbot genome [20], we found only one type II IFN gene, corresponding to *ifng*. To get insights into its functions, we analyzed its constitutive expression and gene modulation after viral and bacterial challenge, as well as its induction by type I IFNs. The bioactivity of turbot Ifng was measured by analyzing its protection capabilities against infections and the induction of specific immune-related genes *in vivo*. Although no protection was observed against bacterial or viral infections, our results showed the dual role of turbot Ifng in the expression of immune genes depending on the pathogen. Ifng seemed to synergistically induce inflammation and type I IFN synthesis upon viral infection, but a reduction in macrophage-related molecules was observed. Strikingly, the opposite effect was observed during the infection with bacteria.

2. Material and Methods

2.1. Characterization of turbot Ifng

The complete sequence of the *ifng* gene was retrieved from the turbot genome project [20]. The open reading frame (ORF) was confirmed by PCR using specific primers and subsequent linking into pCR™2.1-TOPO® vector (Invitrogen) for its cloning using One Shot® TOP10F´ competent cells (Invitrogen) following the protocol instructions. cDNA sequencing was conducted using an automated ABI 3730 DNA Analyzer (Applied Biosystems, Inc. Foster City, CA, USA). The primers used for ORF confirmation are listed in Supplementary Data Table 1. The presence of interferon-gamma activated sites (GAS) and interferon-stimulated response elements (ISRE) was analyzed in the promoter region using the canonical sequences TT(C/A)CNNNAA(A/G) and (G/A)(G/A)AANNGAAA(C/G), respectively.

The presence of signal peptide was analyzed with the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/signalp-3.0/) [21] and the nuclear localization signal (NLS) was determined using NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/) [22]. Molecular weight and isoelectric point were calculated using the Compute pI/Mw tool from ExPASy [23]. The

3D-structure of turbot Ifng was predicted using I-TASSER server [24] selecting the model with the best C-score and viewed by PyMOL (http://www.pymol.org). The Template Modelling Score (TM-score), a measure of structural similarity between two proteins, was also considered in order to identify those structural analogs with known crystal architecture in the Protein Data Bank (PDB; http://www.rcsb.org/pdb/).

2.2. Phylogenetic analysis

IFN-gamma and IFN-gamma-related protein sequences from representative species were retrieved from Genbank (Supplementary Data Table 2). MAFFT online server was used to generate a starting alignment following the E-INS-i strategy [25], which was pruned using Gblocks server 0.91b [26]. The best-fitting amino acid replacement model was determined using ProtTest 3.2 [27] based on the Akaike Information Criterion (AIC) [28]. Finally, the maximum likelihood gene tree was estimated with PhyML 3.0 [29]. Nodal confidence was calculated using 1000 non-parametric bootstrap replicates and represented in a 100 scale in FigTree v1.3.1 [30] (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Fish, virus, bacteria

Juvenile turbot (average weight 2.5 g) were obtained from a commercial fish farm (Insuiña S.L., Galicia, Spain). Animals were maintained in 500 L fibreglass tanks with a re-circulating saline water system (total salinity about 35 g/L) with a light-dark cycle of 12:12 h at 18 °C and fed daily with a commercial dry diet (LARVIVA-BioMar). Prior to experiments, fish were acclimatized to laboratory conditions for 2 weeks. Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number 151-2014.

Viral Hemorrhagic Septicemia Virus (VHSV) strain UK-860/94 was propagated in EPC (Epithelioma Papulosum Cyprini) cells using Eagle's minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS), penicillin and streptomycin (P/S) at 15 °C. The second passage was stored at -80 °C until use.

Aeromonas salmonicida subsp. *salmonicida* (strain VT 45.1 WT) was cultured in tryptic soy agar (TSA) plates at 22 ºC during 24 h before being used.

2.4. Expression plasmid pMCV1.4-*ifng*

The expression plasmid encoding the turbot Ifng, pMCV1.4-*ifng*, was produced using the nucleotide sequence encoding the Ifng mature peptide. The expression (pMCV1.4-*ifng*) and empty plasmid (pMCV1.4) were propagated using One Shot TOP10F´ competent *E. coli* cells (Invitrogen) and the purification was conducted using the PureLinkTM HiPure Plasmid Midiprep Kit (Invitrogen). The expression plasmids encoding turbot type I IFNs (pMCV1.4-*ifn1* and pMCV1.4-*ifn2*) were previously produced in the same way [4].

2.5. *ifng* constitutive expression, induction by type I IFNs and modulation after viral and bacterial challenge.

In order to examine the constitutive expression of *ifng*, eleven different tissues (peritoneal exudate cells – PEC–, blood, head kidney, trunk kidney, spleen, gill, liver, intestine, heart, brain, muscle) were obtained from 3 healthy fish (average weight 125 g). After disinfecting the external surface of the fish with 70% ethyl alcohol, PEC were obtained by the injection of sterile PBS (500 µl) in the peritoneal cavity by using 1 ml syringes with a 25 gauge needle and, after massaging for 10 min, the cell suspensions were recovered. Blood samples were taken from the caudal vein by using a heparinized syringe. In both cases the samples were centrifuged at 1,500 g for 10 min and the supernatants were discarded. All the samples were processed for the analysis of *ifng* expression (section 2.7).

Stimulations with the plasmids encoding two turbot type I IFNs (*ifn1* and *ifn2*) were conducted by intramuscular (i.m.) injection in the region near the caudal peduncle by using 1 ml insulin syringes with a 30 gauge needle and positioned with a 45° insertion angle. Four groups of 6 turbot each were injected with a volume of 50 µl of one of the following treatments: 2.5 µg of pMCV1.4-*ifn1*, 2.5 µg of pMCV1.4-*ifn2*, 2.5 µg of pMCV1.4 (empty plasmid), and phosphate buffered saline (PBS) to the control group. At 48 h, the individuals were sacrificed and muscle (site of plasmid injection) and head kidney were sampled and processed for the analysis of *ifng* expression (section 2.7).

Four groups, composed of 36 turbot/each, were intraperitoneally (i.p.) injected with 50 µl of one of the following treatments: VHSV suspension (5×10^5 $TCID_{50}$/fish), *A. salmonicida* subsp. *salmonicida* suspension (5.5×10^5 CFU/fish), viral medium (MEM+2% FBS+P/S) or PBS. The last two groups served as the control groups of the viral and bacterial infections, respectively. The head kidney from 12 individuals

160 belonging to each group was removed at different sampling points (8, 24 and 72 h),
161 obtaining 4 pooled biological replicates (3 fish/replicate) that were processed for the
162 analysis of *ifng* expression (section 2.7).

163 **2.6. Effect of pMCV1.4-*ifng* injection before and after pathogen challenge**

164 The protective ability of the intramuscular administration of the plasmid encoding Ifng
165 was tested against VHSV and *A. salmonicida*. For the viral challenge, 160 fish were
166 subdivided into 8 batches of 20 turbot each. Turbot from two tanks (two replicates per
167 treatment) were then i.m. injected, in the same way as was done in the previous section,
168 with a volume of 50 µl of one of the following treatments: 2.5 µg of pMCV1.4-*ifng*, 2.5
169 µg of pMCV1.4 (empty plasmid) and PBS. After 2 days, the individuals were i.p.
170 injected with a dose of VHSV of 5×10^5 $TCID_{50}$/fish. The two remaining groups were
171 first i.m. inoculated with PBS and then i.p. with the viral medium and served as an
172 absolute control (non-immunised and non-infected groups). The same experimental
173 procedure was conducted with the Gram-negative bacterium *A. salmonicida* using a
174 dose of 5×10^6 CFU/ml and the corresponding control batches were i.p. injected with
175 PBS. Replicate batches were placed alternatively in order to minimize the influence of
176 tank position. Mortality was recorded over a period of 21 days.

177 In parallel, 2 groups of 18 turbot were also injected with 2.5 µg of pMCV1.4-*ifng* or
178 pMCV1.4. After 48 h, muscle (site of plasmid injection) and head kidney from 6 fish
179 were sampled. Then, 6 fish from each batch were i.p. infected with VHSV and other 6
180 with *A. salmonicida*. At 24 h post-challenge head kidney samples were taken (6
181 individual samples). All these samples were processed for the analysis of gene
182 expression (section 2.7).

183 **2.7. RNA extraction, cDNA synthesis and quantitative PCR analysis**

184 Total RNA from the different tissue samples was extracted using the Maxwell 16 LEV
185 simplyRNA Tissue kit (Promega) with the automated Maxwell 16 Instrument in
186 accordance with instructions provided by the manufacturer. The cDNA synthesis was
187 performed with the SuperScript II Reverse Transcriptase (Invitrogen) using 0.5 µg of
188 RNA.

189 The expression profiles of *ifng* and genes related with the activity of Ifng, as well as the
190 quantification of the VHSV glycoprotein or *A. salmonicida*, were determined using real-

time quantitative PCR (qPCR). Specific qPCR primers for turbot genes were designed based on sequences obtained in a 454-pyrosequencing of turbot tissues [31] or in the turbot genome [20] by using the Primer3 program [32]. Primer sequences are listed in Supplementary data Table 1. Their amplification efficiency was calculated using seven serial five-fold dilutions of head kidney cDNA from unstimulated turbot with the Threshold Cycle (C_T) slope method [33] and the identity of the amplicons was confirmed by sequencing. Individual real-time PCR reactions were carried out in 25 µl reaction volume using 12.5 µl of SYBR GREEN PCR Master Mix (Applied Biosystems), 10.5 µl of ultrapure water (Sigma-Aldrich), 0.5 µl of each specific primer (10 µM) and 1 µl of five-fold diluted cDNA template in MicroAmp optical 96-well reaction plates (Applied Biosystems). All reactions were performed using technical triplicates in a 7300 Real-Time PCR System thermocycler (Applied Biosystems) with an initial denaturation (95 °C, 10 min) followed by 40 cycles of a denaturation step (95 °C, 15 s) and one hybridization-elongation step (60 °C, 1 min). Relative expression data were normalized using the *eukaryotic translation elongation factor 1 alpha* (*eef1a*) as reference gene, which is a good candidate for qPCR data normalization in fish and fish cell lines under infection conditions [34-37], and calculated using the Pfaffl method [33].

2.8 Statistical analysis

Expression results were represented graphically as the mean + the standard deviation of the biological replicates. In order to determine statistical differences, data were analyzed with the computer software package SPSS v.19.0 using the Student's t-test.

3. Results

3.1. Characterization of the turbot *ifng* gene and protein analysis

The complete coding region of turbot *ifng*, submitted to GenBank under accession number KX360748, consists of 609 bp encoding a protein of 202 amino acids (aa) (Figure 1A). The first 22 aa belong to the signal peptide and therefore, the mature peptide is composed by 180 residues, with a calculated molecular weight of 20.36 kDa and an isoelectric point of 10.25. Moreover, a nuclear localization signal was identified in the protein sequence. We also examined the tridimensional structure of the turbot

Ifng and, as expected due to its high structural similarity with human IFN-gamma (TM-score = 0.741), it is mainly alpha helical (Figure 1B).

As in other vertebrates, the genomic sequence turbot *ifng* is composed of 4 exons and 3 introns. A detailed analysis of the promoter region (in a range of -1824 bp upstream) revealed that its expression is putatively regulated by three interferon-gamma activated sites (GAS) and one interferon-stimulated response element (ISRE) (Figure 1C).

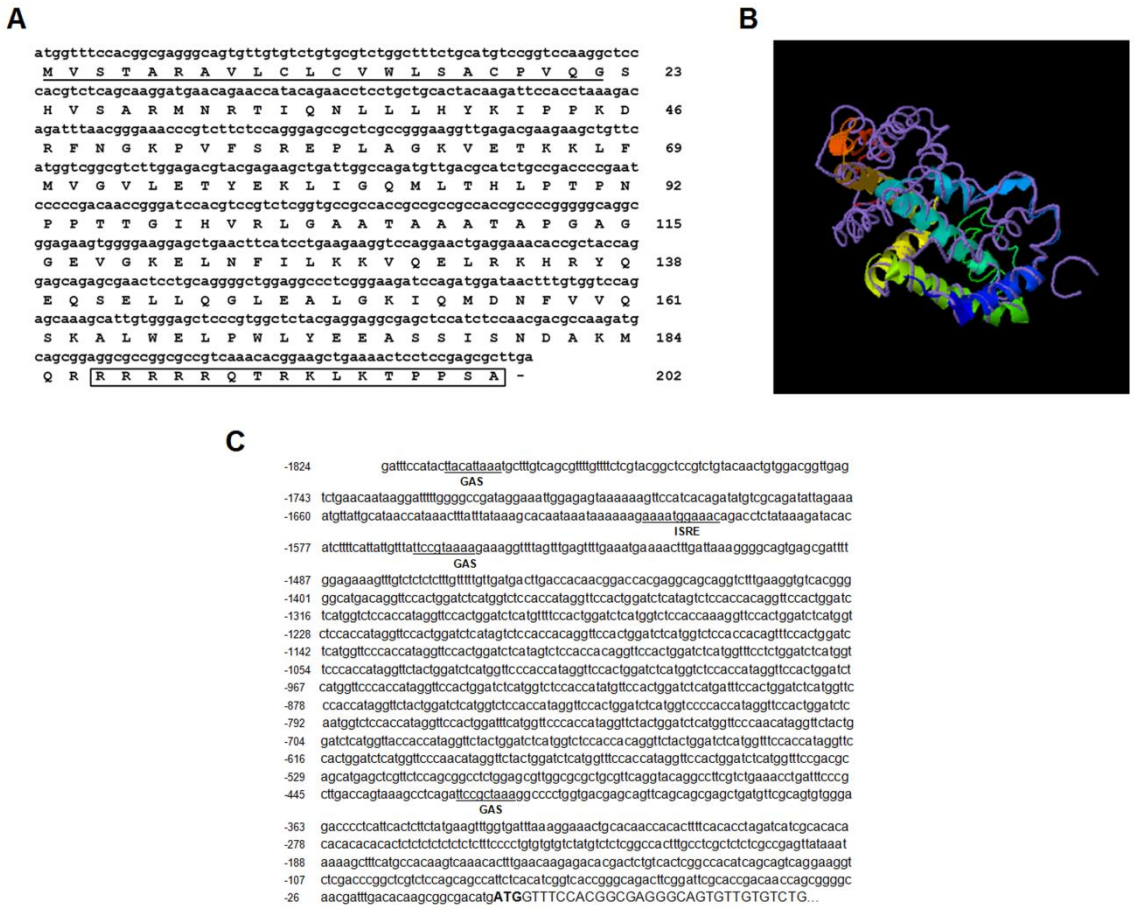

Figure 1. (A) Nucleotide and amino acid sequence of turbot *ifng*. The predicted signal peptide is underlined, whereas the nuclear localization signal (NLS) is boxed. **(B)** Comparison of the predicted tertiary structure of turbot Ifng (color cartoon view) with human IFN-gamma (blue ribbon diagram). **(C)** Promotor region of turbot *ifng* gene showing three potential GAS and one ISRE sequences.

Concurring with the taxonomic classification, the phylogenetic analysis revealed that turbot Ifng was closely related to Ifng proteins from other flatfish species (Figure 2). Moreover, as was expected, Ifngrel proteins formed a separated cluster from the teleost Ifng ones. Nevertheless, teleost Ifng and Ifngrel appear more closely related between them than to vertebrate IFN-gamma proteins (Figure 2).

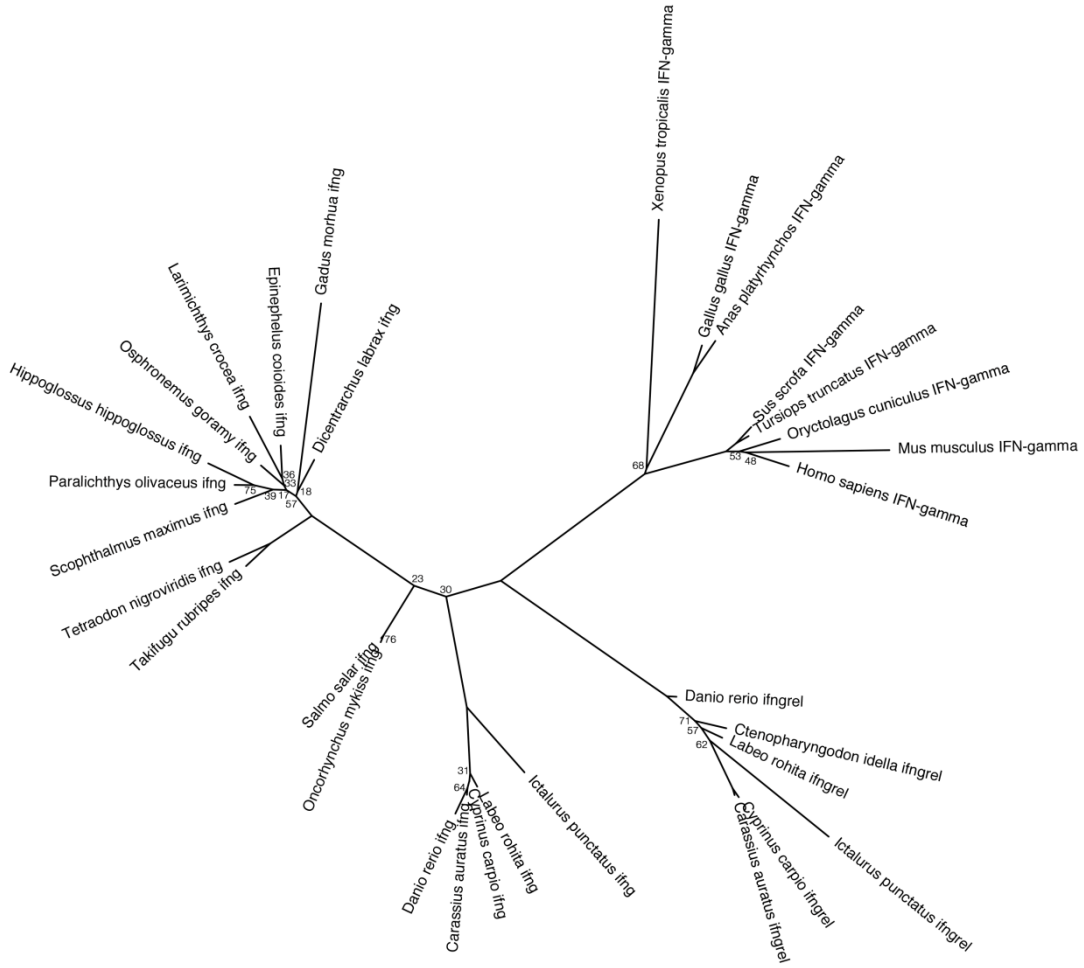

Figure 2. Phylogenetic tree showing the evolutionary relationships between vertebrate IFN-gamma and IFN-gamma related proteins. Only nodal bootstrap values lower than 95 are shown.

3.2. Constitutive expression of *ifng* in different tissues and induction of its transcription

The higher basal expression of turbot *ifng* was detected in PEC, followed by spleen (Figure 3). A similar expression level was observed in gill, trunk kidney and head kidney. The remaining tissues showed a lower *ifng* transcription.

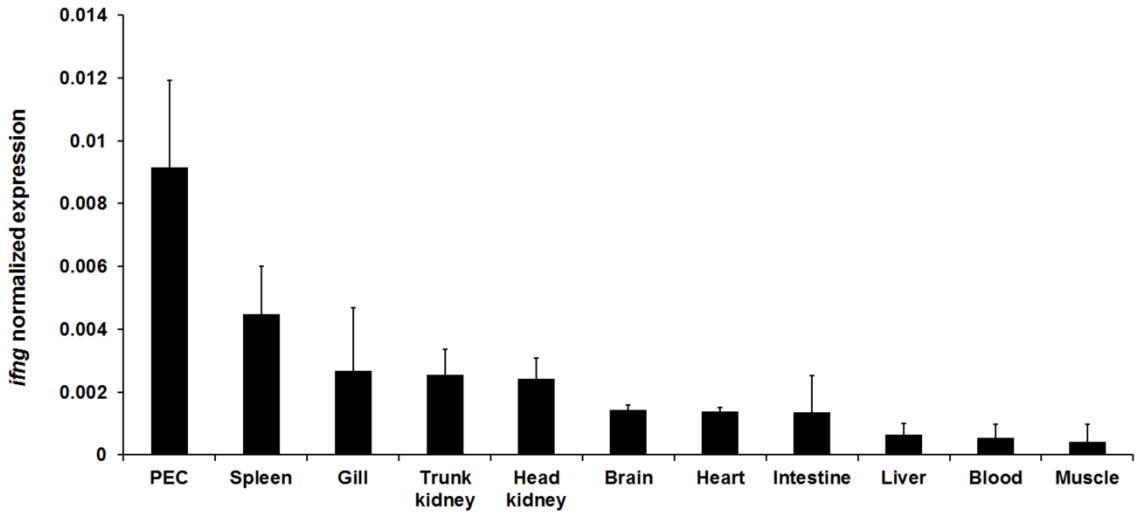

Figure 3. Turbot *ifng* constitutive expression in different tissues obtained from healthy fish.

The i.m. administration of the two expression plasmids encoding type I IFNs (*pMCV1.4-ifn1* and *pMCV1.4-ifn2*), revealed that Ifn1 was able to significantly induce the expression of *ifng* both in muscle (Fold-Change = 2.5) and in head kidney (FC=3.4), whereas Ifn2 induced a significant increase only in the site of injection (FC=1.4) (Figure 4A).

Notably, turbot *ifng* was highly modulated during viral and bacterial challenges (Figure 4B). The i.p. injection of VHSV significantly induced the expression of this gene at 24 (FC=233) and 72 hours post-infection (hpi) (FC=155). *A. salmonicida* challenge also induced significant modulation of *ifng*: higher expression was already detected early at 8 hpi (FC=20.5), which decreased at 24 hpi (FC=4.2), and rose again to its highest expression at 72 hpi (FC=131).

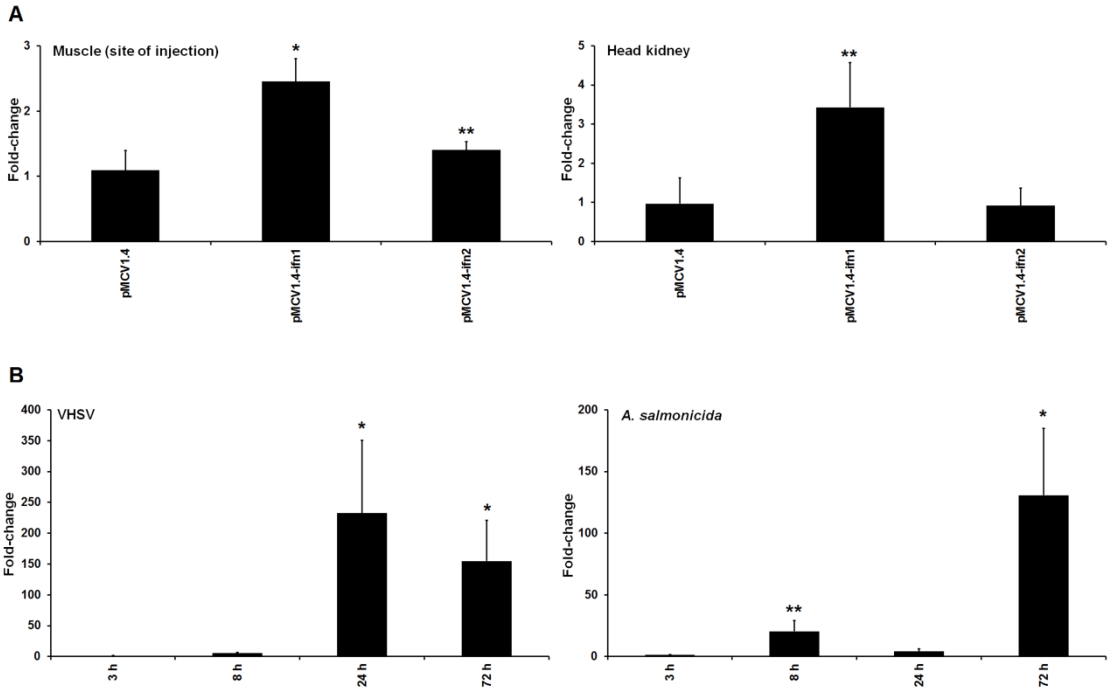

Figure 4. (**A**) Induction of the expression of turbot *ifng* 2 days after the administration of the expression plasmids encoding both type I IFNs (pMCV1.4-*ifn1* or pMCV1.4-*ifn2*) in muscle (site of injection) and head kidney or (**B**) at different sampling points after a VHSV or *A. salmonicida* challenge in head kidney samples. Significant differences are displayed as ** ($0.001<p<0.01$), * ($0.01<p<0.05$).

3.3. *ifng* expression plasmid (pMCV1.4-*ifng*) does not protect turbot against VHSV or A. *salmonicida* infection

Local overexpression of *ifng* was induced with the i.m. injection of 2.5 µg of pMCV1.4-*ifng*, and the increased expression was confirmed 48 hours post-inoculation (Figure 5A). Despite having a 470-fold higher expression of *ifng* than control individuals inoculated with the empty plasmid, pMCV1.4-*ifng* stimulated fish showed a similar pathogen proliferation during early infection stages and not significant differences were observed in the survival rates against VHSV (Figure 5B) and *A. salmonicida* (Figure 5C).

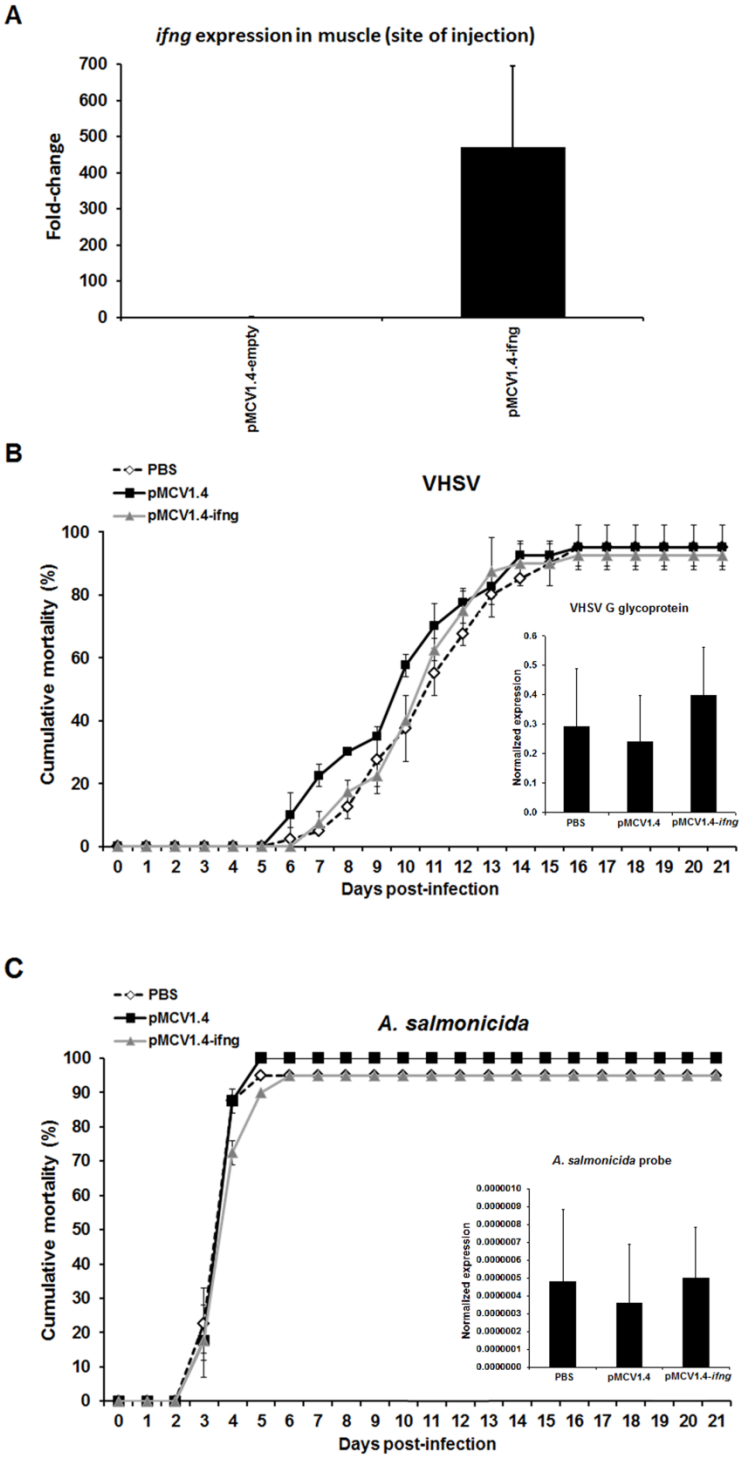

Figure 5. (**A**) Expression of turbot *ifng* in muscle (site of injection) 2 days after the administration of pMCV1.4-*ifng* compared to those individuals receiving the empty plasmid pMCV1.4. (**B**) Cumulative mortality (%) after a VHSV challenge in turbot previously injected in muscle with PBS, pMCV1.4 or pMCV1.4-*ifng*, and VHSV detection by qPCR in head kidney samples 24 h after infection. (**C**) Cumulative mortality (%) after an *A. salmonicida* challenge in turbot previously injected in muscle with PBS, pMCV1.4 or pMCV1.4-*ifng*, and bacteria detection by qPCR in head kidney samples 24 h after infection.

3.4. Pathogen-dependent role of Ifng in macrophage-related molecules expression, type I IFNs induction, and inflammation

At 48 hours after the administration of the expression plasmid pMCV1.4-*ifng*, the modulation of sixteen immune-related genes typically related with the IFN-gamma activity was analyzed in head kidney samples by qPCR. Interestingly, only *macrophage-colony stimulating factor* (*csf1*) was found to be significantly overexpressed (FC=3.33) compared to the pMCV1.4-injected individuals (Figure 6).

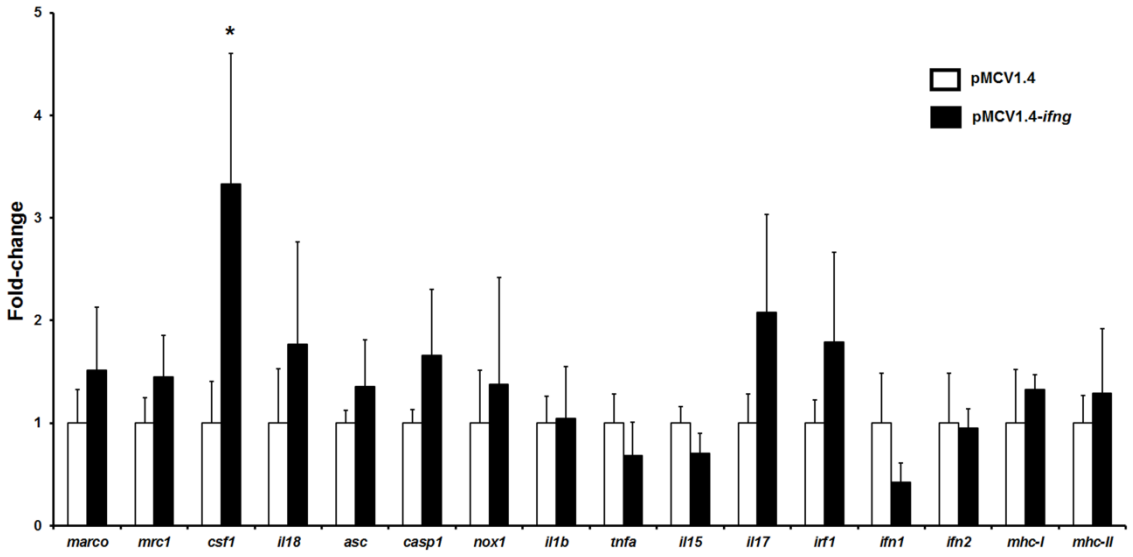

Figure 6. Expression analysis of several immune genes in head kidney samples 2 days after the i.m. injection of the expression plasmid pMCV1.4-*ifng*. Fold-changes were calculated by dividing the normalized expression values for the pMCV1.4-*ifng* inoculated turbot by the normalized expression values for the pMCV1.4- injected individuals (control group). Significant differences were displayed as * ($p<0.05$).

Nevertheless, the most surprising results on the effect of *ifng* overexpression were observed during the viral and bacterial infections. VHSV challenge (pMCV1.4 – VHSV) significantly induced the expression of several immune-related genes compared to non-infected fish: *pyd and card domain containing* (*asc*), *caspase-1* (*casp1*), *interleukin-1beta* (*il1b*), *interleukin-15* (*il15*), *interferon regulatory factor 1* (*irf1*), *ifn1* and *ifn2*, and *major histocompatibility complex- class II* (*mhc-II*) (Figure 7A). Interestingly, *ifng* (pMCV1.4-*ifng* – VHSV fish) showed a synergic induction of immune-related genes compared to pMCV1.4 – VHSV fish (Figure 7A): *il1b* (from FC=6.5 only with virus to FC=17.96 with Ifng and virus), *il15* (from FC=1.75 to FC=4.53), *interleukin-17* (*il17*) (from FC=1.64 to FC=14.15), *ifn1* (from FC=29.43 to FC= 3256) and *ifn2* (from FC=5 to FC=52.36). On the other hand, *ifng* overexpression

also had a detrimental effect in the expression of *macrophage receptor with collagenous structure (marco)*, *csf1*, *nadph oxidase 1 (nox1)* and *mhc-II* in VHSV-infected fish, significantly reducing their expression compared to the pMCV1.4 – VHSV individuals (Figure 7A). In order to discard the potential migration of macrophages to the site of injection of the expression plasmid pMCV1.4-*ifng*, the transcription of *marco* was analyzed in the muscle samples, but no significant differences were observed compared to the control individuals (data not shown).

After *A. salmonicida* challenge, numerous genes were also overexpressed (*csf1*, *asc*, *casp1*, *il1b*, *il17*, *irf1*, and *mhc-II*) (Figure 7B). Strikingly, in this case, the expression of some genes which were potentiated by Ifng during a viral infection was found to be down-regulated by *ifng* overexpression: *il1b* (from FC=28.56 only with bacteria to FC=8.09 with Ifng and bacteria), *il17* (from FC=5.82 to FC=0.97) and *ifn1* (from FC=2.3 to FC=0.54), together with other genes such as *casp1*, *irf1* and *major histocompatibility complex- class I* (*mhc-I*) (Figure 7B). On the contrary, *marco* and *macrophage mannose receptor 1* (*mrc1*) were significantly more expressed in those individuals previously injected with pMCV1.4-*ifng* (Figure 7B).

In order to facilitate the comprehension of all these results, a schematic representation is provided (Figure 7C). The first boxes indicate the effect of a VHSV or *A. salmonicida* challenge in the expression of the tested genes. The second part indicates the effect of the previous administration of Ifng in the gene modulation during a viral or bacterial infection compared to those individuals not receiving the expression plasmid.

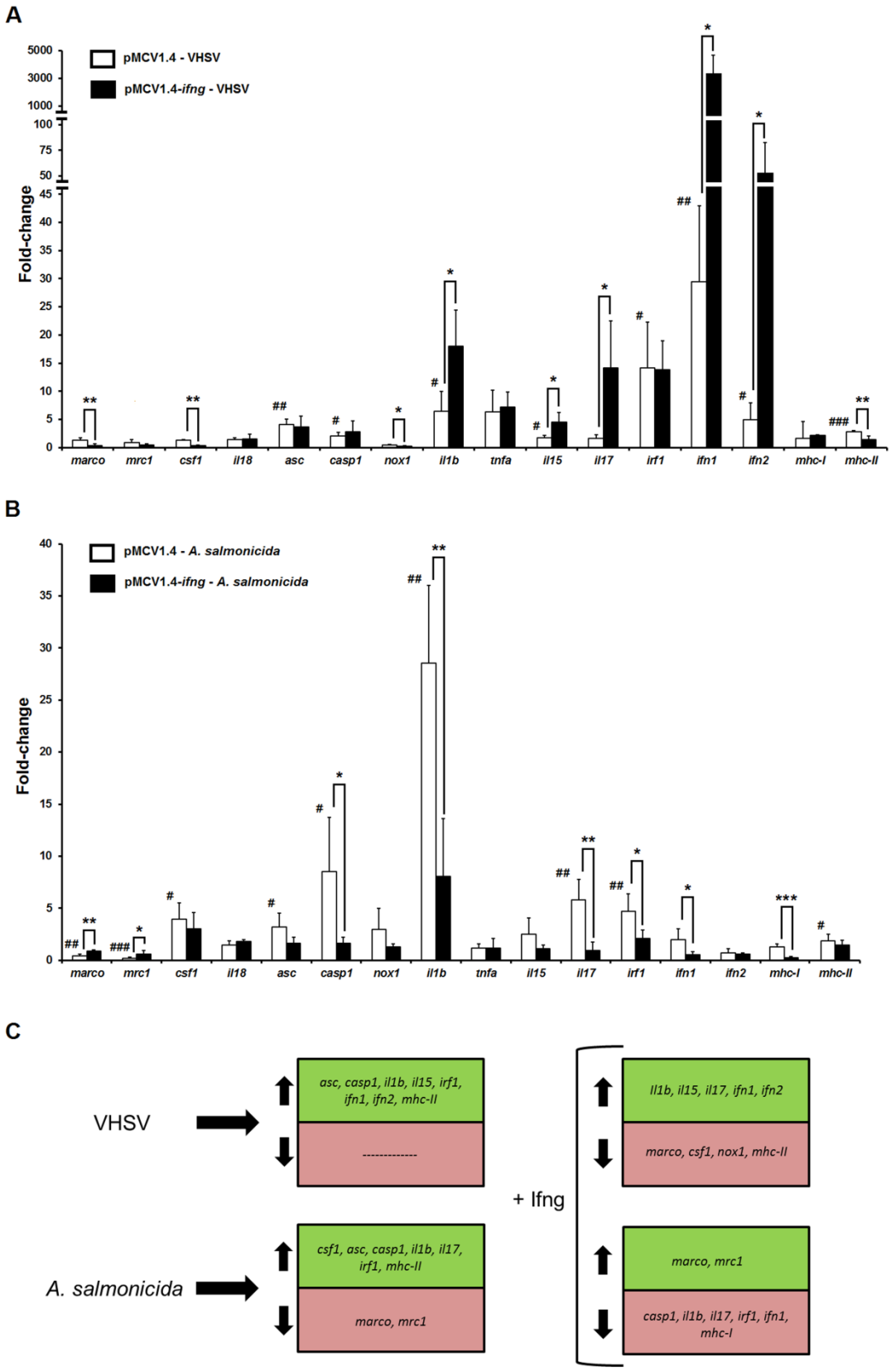

Figure 7. Expression analysis of immune genes in head kidney samples 24 h after the infection with VHSV (**A**) or *A. salmonicida* (**B**) in fish previously injected with the empty plasmid (pMCV1.4) or the expression plasmid (pMCV1.4-*ifng*). Fold-changes were calculated by dividing the normalized expression values for the infected turbot by the normalized expression values for the uninfected pMCV1.4- injected individuals (control group). ###

(0.0001<p<0.001), ## (0.001<p<0.01) and # (0.01<p<0.05) represent gene modulations due to the effect of the infection itself (e.g. pMCV1.4-VHSV and pMCV1.4-*A. salmonicida* vs. pMCV1.4). *** (0.0001<p<0.001), ** (0.001<p<0.01) and * (0.01<p<0.05) represent differences between individuals previously receiving the expression plasmid (pMCV1.4-*ifng*) or the empty plasmid (pMCV1.4) and then infected (**C**) Schematic representation of the significant modulations observed in this experiment.

4. Discussion

Since the first teleost *ifng* gene was detected in the fugu (*Takifugu rubripes*) genome several years ago [38] *ifng* genes were characterized in numerous fish species. However, the absence of sequences for this gene in turbot hindered its identification until now. The turbot (*S. maximus*) genome, recently sequenced [20], has led us to the first-time identification of an *ifng* gene in this flatfish. The additional fish type II IFN (*ifngrel*), mainly characterized in numerous cyprinid species [10,12,14,39], appears to be absent in turbot, as was observed in other teleosts. *ifng* gene encodes a protein composed of 202 aa, and contains a 22 aa signal peptide on the N-terminus and a nuclear localization signal (NLS) near its C-terminus, which is needed for the translocation into the nucleus and the bioactivity of this molecule [40].

Three GAS and one ISRE sequences were found on the promotor region of the turbot *ifng*, which indicate that this gene can be activated via the interferon-gamma- or type I IFNs-induced Jak-Stat signaling pathway [41]. Indeed, turbot *ifng* expression was significantly induced by the i.m. administration of the two expression plasmids encoding Ifn1 and Ifn2, two different type I IFNs. Agreeing with previous studies about the activity of turbot type I IFNs [4], Ifn1 showed a higher and broader activation of *ifng* transcription, whereas Ifn2 activity was localized to the site of injection. *ifng* was also overexpressed after pathogen challenge with both virus and bacteria. Type I IFNs are the main cytokines orchestrating the antiviral defense through the induction of numerous interferon-stimulated genes (ISGs) [6]. However, whereas type I IFNs are pivotal in acute infections, IFN-gamma also contributes to the defense against viruses, but especially during long-term infections [42]. Knockout murine models revealed that the absence of IFN-gamma or the corresponding receptors generates deficiencies in natural resistance to different viruses [43-47]. Deficiencies in the immune response were also observed against bacterial infections [48-52]. Therefore, IFN-gamma seems to be important in the correct resolution of both viral- and bacterial-caused diseases. Nevertheless, the injection of an expression plasmid encoding turbot Ifng was not able

to reduce the mortality induced by VHSV or *A. salmonicida* infections, although we cannot rule out that lower doses of pathogens could reveal any significant difference. Moreover, only *csf1* gene was significantly up-regulated in head kidney 2 days after the administration of pMCV1.4-*ifng*. Similar results were observed in adult zebrafish injected with recombinant Ifng; this molecule had a weak effect on the expression of immune genes and did not reduce the mortality caused by bacterial (*Streptococcus iniae*) or viral (SVCV) infections [18]. Grayfer and Belosevic [53] hypothesized that this lack of response could be due to the fact that Ifng is bounded up by cells expressing only one interferon-gamma receptor (Ifngr1) and not both receptors (Ifngr1 and Ifngr2), indicating a localized nature of the zebrafish Ifng. The majority of experiments performed in mammals and fish using recombinant IFN-gamma were conducted *in vitro* and, in these cases, significant immune effects were observed, especially in macrophages and neutrophils [13,16,17,19,54-57]. Probably the immune effects of recombinant Ifng *in vivo* are more subtle and dependent on the doses and protocols of inoculation, among others.

After infection with VHSV some immune genes were significantly higher expressed in those individuals previously inoculated with the *ifng* expression plasmid than in those injected with the empty plasmid. These genes corresponded to pro-inflammatory cytokines (*il1b, il15, il17*) and both type I IFNs (*ifn1* and *ifn2*). Surprisingly, on the contrary, *il1b, il17* and *ifn1* (together with *casp1*, *irf1* and *mhc-I*) were lower expressed after an *A. salmonicida* challenge in those individuals previously stimulated with pMCV1.4-*ifng*. Therefore, although pMCV1.4-*ifng* did not elicit a protective effect in VHSV- or *Aeromonas*-infected turbot, its i.m. injection was able to specifically modulate the expression of different pro-inflammatory cytokines and type I IFNs in an opposite way depending on the pathogen.

Classically, IFN-gamma has been regarded as a pivotal pro-inflammatory cytokine in inflammation and autoimmune diseases [9]. However, during the last two decades numerous evidences supported the idea of a dual role of IFN-gamma genes in inflammation [8, 9], highlighting the complexity of the immune mechanisms in which this molecule is implicated. Although type I IFNS are the main anti-viral signaling molecules, IFN-gamma also seems to be essential in the resolution of viral diseases [43-47]. The activity of IFN-gamma includes the activation of a subset of IFN-gamma inducible genes, such as the transcription factor IRF1 [58], which is able to activate type

I IFN gene promoters and induce type I IFN genes [59]. Therefore, in the case of VHSV infection, the effect of *ifng* overexpression could be expected, acting in synergy with the virus and potentiating the inflammation and expression of type I IFNs. Regarding the effect of IFN-gamma in bacterial infections, the observations conducted in murine models are contradictory, attributing to this protein a pro-inflammatory [60,61] or anti-inflammatory role [62,63] depending on the bacteria and experimental design.

MARCO and MRC1 are receptors typically expressed in macrophages and implicated in the clearance of pathogens [64-66]. The transcription of *marco* and *mrc1* was significantly down-regulated after bacterial infection but restored in those fish also receiving the expression plasmid encoding Ifng. The increase in the transcription of *marco* and *mrc1* by Ifng seems to indicate a positive effect in the macrophages activity during bacterial infection. In contrast, the opposite behavior was observed during VHSV infection, in which Ifng reduced the transcription of *marco*, *csf1* and *nox1*. *csf1* is implicated in the proliferation of macrophages [67], and *nox1* is an enzyme mediating the respiratory burst in macrophages and neutrophils, a potent microbicidal mechanism [68]. Therefore, the reduction in the expression of these genes could be reflecting that Ifng exerts a suppressive effect in the macrophages activity when the individuals are suffering a viral infection.

It has been reported that macrophages are probably the target cells of the rhabdovirus infections, at least during the first stages of infection, and that they are destroyed through virus-induced pyroptosis [69,70]. Due to the effect of Ifng during VHSV infection (down-regulation of *marco* and *csf1*, and overexpression of pro-inflammatory genes), we thought about the possibility that Ifng was favoring the death of the macrophages. For that reason, we checked the transcriptomic modulation of *asc* and *casp1*, two genes encoding for key components of the pyroptosis process. However, no significant increases in the expression of both genes were observed in those turbot pre-treated with pMCV1.4-*ifng* compared to the individuals injected with the empty plasmid. Therefore, Ifng could be directly modulating the expression of *marco* and *csf1*. It was reported that mutant mice deficient in MARCO (MARCO –/–) had lower morbidity and mortality caused by influenza pneumonia than wild-type mice because MARCO suppresses the early inflammatory response against the virus [71]. Therefore, if Ifng reduces the expression of *marco* in turbot during a viral infection, the higher level of pro-inflammatory cytokines could be due to the effect of Marco in the

inflammatory response. This is also in accordance with the activity of Ifng during the bacterial challenge, because *marco* was overexpressed in pre-treated fish compared to the individuals only inoculated with the bacteria, and the pro-inflammatory cytokines were down-regulated in this case. We hypothesize that *marco* could be directly modulated by Ifng and, as consequence, this can have an effect in the inflammation.

The innate immune system's recognition of pathogens is predominantly mediated by a limited range of genome-coded pattern recognition receptors (PRRs). PRRs are activated by the recognition of pathogen-associated molecular patterns (PAMPs), such as bacterial and fungal glycoproteins and lipopolysaccharides or viral components [2]. Once activated by the recognition of its specific ligand, PRRs initiate intracellular signaling cascades with the objective to produce an adequate response to the stimulus that activated them [72]. The specific inflammatory response is both dependent on the PAMP that was detected and the cell type that was activated, that is, viral PAMPs are recognized by different PRRs than those recognizing bacterial components and use different intracellular pathways that lead to the transcription of different inflammatory genes and the activation of different immune cell subsets [2]. This differential activation of the cellular immune response, together with the fact that each pathogen possesses preferences for its own target cells, culminates in a pathogen-specific immune response [73]. Although more research is needed, the dual role of Ifng that we demonstrate in this article could be due to the differential effect that this molecule exerts on the different already activated immune cell populations.

5. Conclusions

We can resume all these results into three main points: I) Ifng potentiated inflammation during viral infection, but it had anti-inflammatory effects during bacterial disease; II) Ifng administration had a synergic or detrimental effect in the transcription of type I IFNs depending on the pathogen; III) Ifng seemed to favor the expression of those genes directly related with the activity of macrophages in *A. salmonicida*-infected turbot, but the opposite effect was observed in VHSV-infected individuals.

Further research is needed in order to elucidate these gene regulations, probably by using a model organism such as the zebrafish. Given that in this study viral and

bacterial infections were conducted in parallel, using the same experimental design and cohort of fish, this is the first time that the dual role of IFN-gamma has been clearly exposed.

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FIGURE CAPTIONS

Figure 1. (A) Nucleotide and amino acid sequence of turbot *ifng*. The predicted signal peptide is underlined, whereas the nuclear localization signal (NLS) is boxed. **(B)** Comparison of the predicted tertiary structure of turbot Ifng (color cartoon view) with human IFN-gamma (blue ribbon diagram). **(C)** Promotor region of turbot *ifng* gene showing three potential GAS and one ISRE sequences.

Figure 2. Phylogenetic tree showing the evolutionary relationships between vertebrate IFN-gamma and IFN-gamma related proteins. Only nodal bootstrap values lower than 95 are shown.

Figure 3. Turbot *ifng* constitutive expression in different tissues obtained from healthy fish.

Figure 4. (**A**) Induction of the expression of turbot *ifng* 2 days after the administration of the expression plasmids encoding both type I IFNs (pMCV1.4-*ifn1* or pMCV1.4-*ifn2*) in muscle (site of injection) and head kidney or (**B**) at different sampling points after a VHSV or *A. salmonicida* challenge in head kidney samples. Significant differences are displayed as ** ($0.001<p<0.01$), * ($0.01<p<0.05$).

Figure 5. (**A**) Expression of turbot *ifng* in muscle (site of injection) 2 days after the administration of pMCV1.4-*ifng* compared to those individuals receiving the empty plasmid pMCV1.4. (**B**) Cumulative mortality (%) after a VHSV challenge in turbot

previously injected in muscle with PBS, pMCV1.4 or pMCV1.4-*ifng*, and VHSV detection by qPCR in head kidney samples 24 h after infection. (**C**) Cumulative mortality (%) after an *A. salmonicida* challenge in turbot previously injected in muscle with PBS, pMCV1.4 or pMCV1.4-*ifng*, and bacteria detection by qPCR in head kidney samples 24 h after infection.

Figure 6. Expression analysis of several immune genes in head kidney samples 2 days after the i.m. injection of the expression plasmid pMCV1.4-*ifng*. Fold-changes were calculated by dividing the normalized expression values for the pMCV1.4-*ifng* inoculated turbot by the normalized expression values for the pMCV1.4- injected individuals (control group). Significant differences were displayed as * ($p<0.05$).

Figure 7. Expression analysis of immune genes in head kidney samples 24 h after the infection with VHSV (**A**) or *A. salmonicida* (**B**) in fish previously injected with the empty plasmid (pMCV1.4) or the expression plasmid (pMCV1.4-*ifng*). Fold-changes were calculated by dividing the normalized expression values for the infected turbot by the normalized expression values for the uninfected pMCV1.4- injected individuals (control group). ### ($0.0001<p<0.001$), ## ($0.001<p<0.01$) and # ($0.01<p<0.05$) represent gene modulations due to the effect of the infection itself (e.g. pMCV1.4-VHSV and pMCV1.4-*A. salmonicida* vs. pMCV1.4). *** ($0.0001<p<0.001$), ** ($0.001<p<0.01$) and * ($0.01<p<0.05$) represent differences between individuals previously receiving the expression plasmid (pMCV1.4-*ifng*) or the empty plasmid (pMCV1.4) and then infected (**C**) Schematic representation of the significant modulations observed in this experiment.

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