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Cytokine-like activity of European sea bass ISG15 protein on RGNNV-infected E−11 cells

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ABSTRACT

IFN-I generates an antiviral state by inducing the expression of numerous genes, called IFN-stimulated genes, ISGs, including ISG15, which is the only ISG with cytokine-like activity. In a previous study, we developed the Dl_ISG15_E11 cell line, which consisted of E11 cells able to express and secrete sea bass ISG15. The current study is a step forward, analysing the effect of secreted sea bass ISG15 on RGNNV replication in E11 cells, and looking into its immunomodulatory activity in order to corroborate its cytokine-like activity. The medium from ISG15-producing cells compromised RGNNV replication, as it has been demonstrated both, by reduction in the viral genome synthesis and, specially, in the yield of infective viral particles. The implication of sea bass ISG15 in this protection has been demonstrated by ISG15 removal, which decreased the percentage of surviving cells upon viral infection, and by incubation of RGNNV-infected cells with a recombinant sea bass ISG15 protein, which resulted in almost full protection. Furthermore, the immunomodulatory activity of extracellular sea bass ISG15 has been demonstrated, which reaffirms a cytokine-like role for this protein.

1. Introduction

Interferon-stimulated gene 15 (*isg15*) is one of the interferon-stimulated genes (ISGs) within the type-I interferon system (IFN–I). It encodes a 15-kDa protein composed of two ubiquitin-like domains and a C-terminal RLRGG motif. Within producing cells, this protein can be found either free or conjugated to viral or cellular proteins. Conjugation to target proteins, called ISGylation, is a reversible process similar to ubiquitination that requires the C-terminal motif and results in the modification of some characteristics of the conjugated proteins [1].

In mammals, ISG15 can be secreted, acting as a cytokine able to attract or stimulate immune cells [2]. ISG15-releasing cells include leukocytes, monocytes, fibroblasts, epithelial cells and neutrophils [3–5]. The mechanism underlying ISG15 secretion is unknown, and its receptor has only been described for human natural killer (NK) cells. On those cells, ISG15 promotes IFN-γ and interleukin-10 synthesis [6]. Other functions reported for secreted ISG15 in mammals are stimulation of NK proliferation, stimulation of dendritic cell maturation, attraction of neutrophils, induction of IFN-γ production by NK and lymphocytes T, as well as activation of monocytes and macrophages via IFN-γ [7].

In fish, *isg15* transcription is strongly upregulated very early after viral and bacterial infections [8–17], and ISG15 proteins restrict the replication of both, RNA and DNA viruses [18–22]. As it has been described for mammals, this protein can be secreted by fish immune cells, having immunomodulatory properties on head kidney lymphocytes, as it has been described for tongue sole (*Cynoglossus semilaevis*), red drum (*Sciaenops ocellatus*) and Atlantic salmon (*Salmo salar*) ISG15 proteins [10,13,18]. Moreover, ISG15 has also been detected extracellularly in cultures of non-immune cells, such as epithelioma papulosum cyprini cells (EPC) and E11 cells expressing zebrafish (*Danio rerio*) or European sea bass (*Dicentrarchus labrax)* ISG15, respectively [20,21].

ISG15 has multiple roles, and recent studies reveal important functional differences between species [23], which reinforces the necessity of disclosing its role in each virus-host system. The present report is focused on the extracellular activity of European sea bass ISG15 (Dl_ISG15) against nervous necrosis virus (NNV), which causes the viral

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nervous necrosis disease. NNV is a betanodavirus with a genome composed of two single-stranded, positive-sense RNA segments: RNA1, coding for the viral polymerase; and RNA2, encoding the capsid protein. This virus has been classified into four species (former genotypes) [24], although only red-spotted grouper nervous necrosis virus (RGNNV) causes severe disease in European sea bass [25].

In a previous study, we developed the Dl_ISG15_E11 cell line, which consisted of E11 cells constitutively expressing European sea bass ISG15. Dl_ISG15 was detected in the cytoplasm, as well as in the medium of those cells. Furthermore, the activity of Dl_ISG15-containing medium was preliminary evaluated, causing an enhanced E11 survival rate following RGNNV or striped jack nervous necrosis virus (SJNNV) inoculation [21]. The current study is a further insight into the cytokine-like activity of sea bass ISG15, analysing its effect on RGNNV replication, and looking into its immunomodulatory activity. To our knowledge, this is the first report on the effect of an extracellular ISG15 protein on non-immune cells.

2. Materials and methods

2.1. Cell culture and virus

E11 and Dl_ISG15_E11 cell lines [21,26] were cultured at 25 °C with Leibovitz L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), and 100 units/mL penicillin-10 mg/mL streptomycin (Sigma). In order to obtain Dl_ISG15-containing medium (hereafter Dl_ISG15-medium) and Dl_ISG15-free medium (used as control), both cell lines were seeded in 25-cm^2 flasks and incubated for 72 h. Afterwards, supernatants were collected and cellular debris were removed by centrifugation. All experiments were conducted with the same batch of Dl_ISG15-containing and Dl_ISG15-free media, which were stored at −20 °C until used.

The RGNNV isolate SpDl_IAusc965.09, obtained from European sea bass [27], has been propagated and titrated on E11 cells cultured at 25 °C with L-15 medium supplemented with 2% FBS and 100 units/mL penicillin-10 mg/mL streptomycin (maintenance medium). Titration has been carried out according to the 50% tissue culture infective dose ($TCID_{50}$) method [28].

2.2. Production of anti-Dl_ISG15 antibodies

A polyclonal antiserum against a recombinant 6His-tagged Dl_ISG15 protein has been produced. For protein expression, *Escherichia coli* BL21 (DE3) cells were transformed with the pGEX-6P-2 plasmid (GE Healthcare) containing the full-length sea bass *isg15* gene fused to the glutathione S-transferase (GST) gene. Sea bass *isg15* was amplified according to Moreno et al. [15]. Bacteria were cultured in 2xYTA broth (1.6% tryptone, 1% yeast extract and 0.5% NaCl) with ampicillin (100 μg/mL, Sigma) at 37 °C until mid-log phase. Protein expression was then induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) at 37 °C for 2 h. The resulting GST-ISG15 protein was purified using the GST Bulk Kit (GE Healthcare), and GST was subsequently removed with a specific GST PreScision protease (GE Health care). The purified recombinant ISG15 protein (rDl_ISG15) was quantified by Qubit fluorometric analyses (ThermoFisher) and stored at −20 °C until used.

For antibody production, New Zealand white rabbits were injected with rDl_ISG15 (0.5 mg). Booster doses were inoculated 14, 42 and 56 days after the first injection, and animals were bled at 70 days post-first injection. This procedure was performed by ThermoFisher corporation. Antibodies in sera were precipitated with ammonium sulphate, dialyzed against PBS at room temperature overnight, and purified with Nab Protein A Plus Spin Column (Thermo). The specificity of these antibodies was evaluated by Western blot as described by Moreno et al. [21].

2.3. Dl_ISG15 immunoprecipitation

Sea bass ISG15 was pelleted using anti-Dl_ISG15 antibodies (1 μg) and the Protein G system (Roche), according to manufacturer instructions. Immunoprecipitation was verified by immunoblotting [29], using anti-Dl_ISG15 (1:5,000) and goat anti-rabbit IgG-peroxidase (1:10,000, Sigma) antibodies. For peroxidase detection, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and Chem-iDoc-XRS + imagen system (Bio-Rad) were used.

2.4. Cellular survival analyses

Survival analyses involving both, Dl_ISG15-medium and rDl_ISG15, have been performed in order to evaluate the protection of RGNNV-infected E11 cells mediated by ISG15.

For Dl_ISG15-medium analyses, E11 cells were inoculated with RGNNV at 0.05 multiplicity of infection (MOI) in L-15 medium supplemented with 100 units/mL penicillin-10 mg/mL streptomycin. After incubation at 25 °C for 1 h (adsorption time), virus suspension was removed and immediately replaced by Dl_ISG15-medium (serial dilutions) or 1:2-diluted Dl_ISG15-medium after ISG15 immunoprecipitation (ipDl_ISG15).

Regarding rDl_ISG15 analyses, two different approaches have been carried out: (i) cells were exposed to rDl_ISG15 (serial dilutions) or iprDl_ISG15 (0.15 mg/mL) after viral adsorption time, as above described; (ii) cells were treated with 0.15 mg/mL rDl_ISG15 for 48 h before viral inoculation in addition to be exposed to rDl_ISG15 after viral adsorption time.

All dilutions were performed in L-15 maintenance medium. Infected and non-infected cells cultured with ISG15-free medium were always included as controls. All experiments were performed in triplicate.

The percentage of surviving cells was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-reduction assay after 6 days of incubation at 25 °C [21]. Optical density (OD) was measured with the Whittaker Microplate Reader 2001 at 550 nm. Resulting values were normalized by subtracting OD values obtained for L-15 medium. In parallel, cytopathic effects (CPEs) were examined with an Eclipse Ti microscope (Nikon) at 6 days post-infection (dpi).

2.5. Viral replication analyses

E11 cells grown on 24-well plates were inoculated with RGNNV as aforementioned (0.05 MOI). After viral adsorption, virus suspension was removed and immediately replaced by Dl_ISG15-containing or Dl_ISG15-free medium (1:2 in L-15 maintenance medium). Cells were incubated at 25 °C, being harvested in triplicate at 1, 2, 3, 5 and 7 dpi for viral genome quantification. In addition, extracellular viral particles were titrated by the $TCID_{50}$ method at 7 dpi.

Cellular RNA was extracted using the EZNA total RNA Kit, and treated with RNase-free DNase I (Sigma-Aldrich) before cDNA synthesis, which was carried out with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Resulting cDNA was stored at −20 °C until used.

RNA2 viral genomic segment was quantified by absolute real-time PCR according to Moreno et al. [27]. Amplifications were conducted with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix, using cDNA generated from 50 ng RNA. Amplification conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 52 °C for 10 s and 72 °C for 10 s. Primers used are shown in Table 1.

2.6. Immunomodulation analyses

E11 cells seeded on 24-well plates were cultured at 25 °C for 24 h. Afterwards, the medium was removed and immediately replaced by Dl_ISG15-containing or Dl_ISG15-free medium (1:2 in L-15 maintenance medium). Those cells were incubated at 25 °C and sampled in triplicate after 6, 24 and 48 h for RNA extraction and cDNA synthesis, following

Table 1
Primers used in this study.

Target gene	Sequence (5′-3′)	Amplicon size (bp)	Reference
RNA2	F: ACCGTCCGCTGTCTATTGACTA R: CAGATGCCCCAGCGAAACC	126	[16]
mx	F: GGGGTCAGAAGGAGATCACA R: ATGATGCACCAGCTCAAGTG	150	[46]
tlr3	F: TGCAACACTCCACTGACTTACTTTAA R: AGGACAGCTGTGCTAAGTATATAA	115	[21]
tnfα	F: TCCAAGGCAGCCATCCATTT R: TGTTGTTCACCAGCCTGAA	108	This study
hsp70	F: GTCGTGGATCTGTCCCTTGT R: CTCGCTTTGAGGAGCTGTG	98	[47]
actβ	F: CACTGTGCCCATCTACGAG R: CCATCTCCTGCTCGAAGTC	200	[48]

the above-described procedures.

Transcription of toll-like receptor 3 (*tlr3*), tumour necrosis factor (*tnfα*), mx dynamin like GTPase (*mx*), and heat shock protein 70 (*hsp70*) was quantified by relative real-time PCR, using actin-beta subunit (*actβ*) as reference gene and the PCR conditions previously described. Fold change (FC) values were calculated by the comparative delta Ct method [30]. Primers used are shown in Table 1.

In addition, the effect of extracellular ISG15 proteins on *mx* and *tlr3* transcription in the course of RGNNV infections has been evaluated by analysing cDNA samples obtained during the viral replication analyses.

2.7. Statistical analyses

The GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA) was used for data analyses. Normality distribution was verified by the Shapiro-Wilk test. One-way ANOVA was the statistical test used, with Tukey's multiple comparison analysis as post-hoc test. Values of $p < 0.05$ were considered significant.

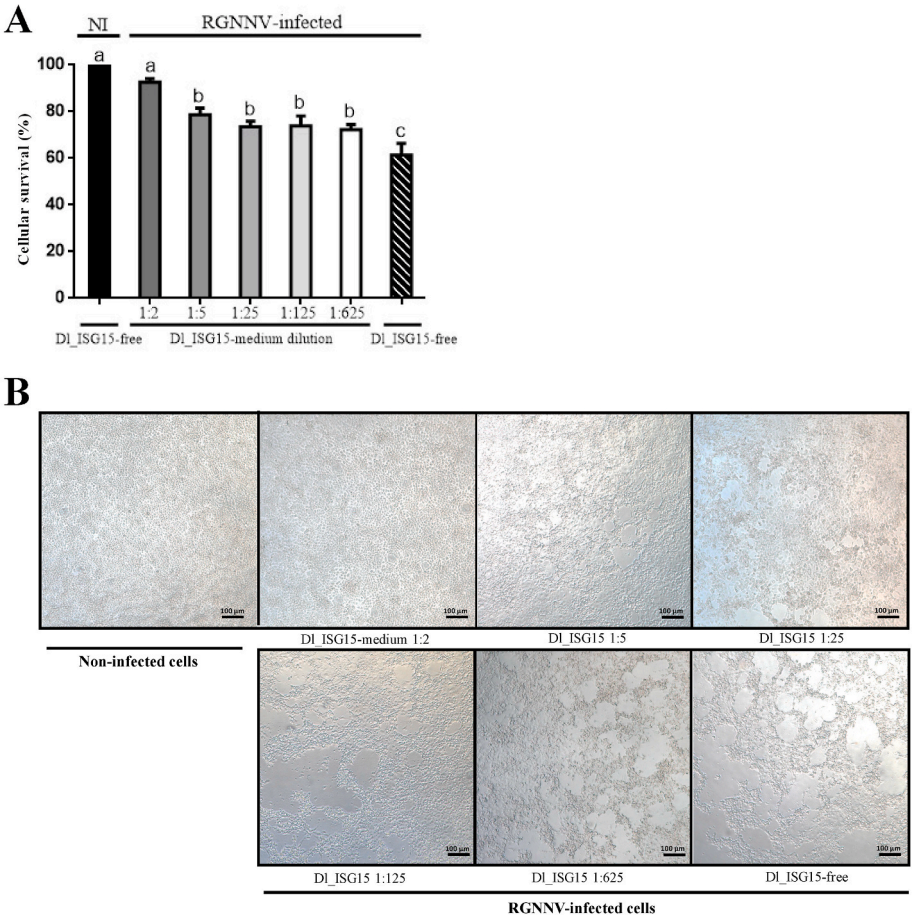

Fig. 1. Dose-dependent effect of extracellular sea bass ISG15 on RGNNV-infected E11 cells. (A) Survival rate of cells exposed to serial dilutions of Dl_ISG15-medium. NI: non-infected. Different letters denote significant differences ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3). (B) CPEs of cells incubated with serial dilutions of Dl_ISG15-medium. Photos were taken at 6 days post-infection.

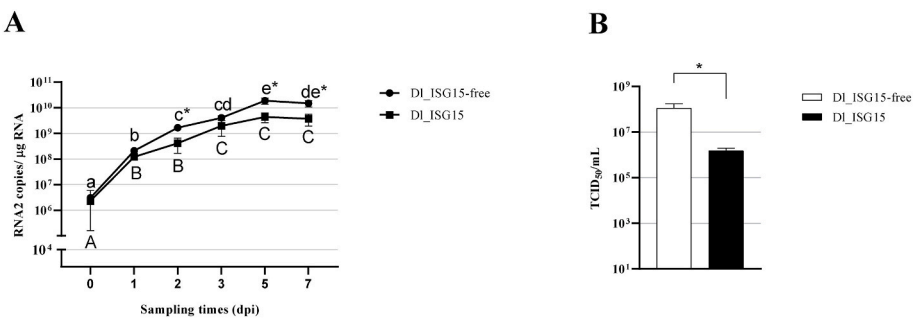

Fig. 2. RGNNV multiplication in E11 cells incubated with Dl_ISG15-containing or Dl_ISG15-free medium. (A) Number of RNA2 copies in cells at different times post-infection. Different letters indicate significant differences throughout the time within each experimental group. Asterisks show significant differences between experimental groups at the same sampling time ($p < 0.05$). (B) Extracellular infective viral particles ($TCID_{50}$/mL) at 7 days post-infection. Asterisk shows significant difference ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).

3. Results

3.1. Dose-dependent protection of extracellular Dl_ISG15

As it is shown in Fig. 1A, 92.6% of cells incubated with 1:2-diluted Dl_ISG15-medium survived RGNNV infection. This survival rate was statistically similar to that obtained for uninfected cells, and significantly higher than that recorded for cells exposed to 1:5-diluted medium (78.7%, $p = 0.001$). The analysis of higher dilutions (up to 1:625) did not change the percentage of surviving cells; however, a clear gradation in CPE severity was observed from cells exposed to the lowest dilution, where CPEs were hardly noticed, to cells incubated with the highest dilution, developing CPEs similar to those observed in untreated infected cells (Fig. 1B).

3.2. Dl_ISG15-medium compromises RGNNV replication

To get a better understanding of the anti-RGNNV activity of secreted sea bass ISG15, viral genome replication was analysed (Fig. 2A). In Dl_ISG15 group, RNA2 copy number reached a plateau at 3 dpi (2×10^9 copies/µg RNA), whereas in control cells viral genome replication was recorded up to 5 dpi, when a maximum value of 1.9×10^{10} copies/µg RNA was recorded (Fig. 2A). In addition, the number of RNA2 copies was significantly lower ($p < 0.05$) in cells exposed to ISG15 from 2 dpi onwards (except for 3 dpi), recording 2.8×10^9 and 1.5×10^{10} copies/µg RNA at 7 dpi for Dl_ISG15 and control groups, respectively (Fig. 2A).

The titre of extracellular viral particles was also significantly lower ($p < 0.05$) in Dl_ISG15 group (1.5×10^6 $TCID_{50}$/mL) than in control group (1.5×10^8 $TCID_{50}$/mL) at 7 dpi (Fig. 2B).

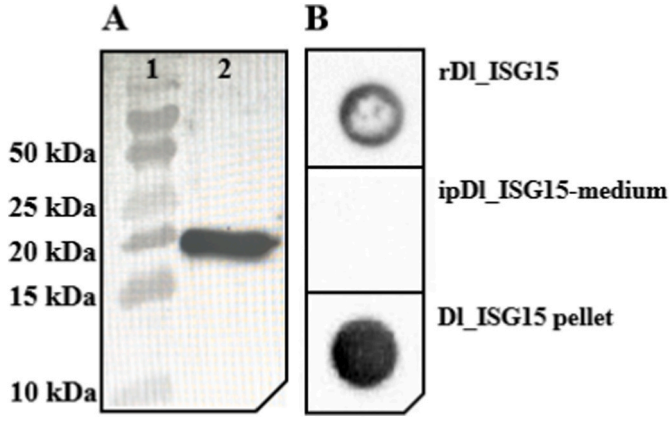

Fig. 3. Detection of sea bass ISG15 using antibodies developed in this study. (A) Western blot. Lane 1: Precision Plus Protein Standard Dual Color (BioRad); lane 2: rDl_ISG15. (B) Dot-blot. Samples analysed were: rDl_ISG15 (positive control); ipDl_ISG15-medium (Dl_ISG15-medium after ISG15 removal); and pellet obtained after ISG15 immunoprecipitation.

3.3. Dl_ISG15 partially accounts for the protection exerted by Dl_ISG15-medium on RGNNV-infected cells

The anti-RGNNV activity of Dl_ISG15-medium may be due to the direct action of ISG15 on target cells, or may be a consequence of other cytokines also secreted by Dl_ISG15-producing cells. In order to clear up this issue, ISG15 molecules were immunoprecipitated using antibodies developed in this study, which recognised the 20-kDa Dl_ISG15 protein expressed in *E. coli* (15-kDa ISG15 plus 6xHis-tag) (Fig. 3A). The immunoblot analysis showed that immunoprecipitation removed sea bass ISG15 from the medium, which was used for cell survival analyses, whereas ISG15 was successfully detected in the pellet, which was discarded (Fig. 3B).

According to Fig. 4A, ISG15 removal resulted in a significant ($p < 0.0001$) decrease in the cellular survival rate, which was 92.6% in Dl_ISG15 group and dropped to 75.2% in ipDl_ISG15 group. However, cells in ipDl_ISG15 group were still partially protected, since the survival rate of untreated infected cells was significantly lower (61.4%, $p < 0.0001$) (Fig. 4A). Therefore, sea bass ISG15 accounts for 47% of the anti-RGNNV activity recorded for Dl_ISG15-medium (Fig. 4B). These results were supported by CPE monitoring, which showed that CPE severity in RGNNV-infected cells exposed to ipDl_ISG15-medium was moderate, whereas cells in Dl_ISG15 and Dl_ISG15-free groups displayed minor and mayor CPEs, respectively (Fig. 4C).

3.4. Recombinant Dl_ISG15 mimics the protection conferred by Dl_ISG15-medium

The direct action of sea bass ISG15 on E11 cells was further evaluated by incubating RGNNV-infected cells with rDl_ISG15 at different concentrations (Fig. 5A). Cells exposed to 1.5×10^{-1} mg/mL were almost fully protected, since 93.1% of these cells survived the infection, whereas a significant decrease in the survival rate was recorded as rDl_ISG15 concentration was decreasing (Fig. 5A). Cells exposed to concentrations lower than 1.5×10^{-3} mg/mL were not protected, showing survival rates statistically similar to that recorded for untreated infected cells (58.6%).

In addition, protection exerted by 1.5×10^{-1} mg/mL rDl_ISG15 was completely abolished after rISG15 immunoprecipitation (Fig. 5B). The percentage of cells incubated with iprDl_ISG15 surviving the infection (52.5%) was statistically similar ($p < 0.001$) to that recorded for untreated infected cells.

3.5. Extracellular Dl_ISG15 modulates immunogene transcription

Transcription of *tnfα*, *mx, tlr3* and *hsp70* has been analysed in control and ISG15-exposed cells (Fig. 6A). *tnfα* transcription was not altered by Dl_ISG15, whereas *mx, tlr3* and *hsp70* transcription was significantly different in both experimental groups at different times (Fig. 6A). Specifically, *mx* was upregulated in Dl_ISG15 group at 6 h (2.0 log_2-FC) and 48 h (6.5 log_2-FC), whereas *tlr3* and *hsp70* were downregulated (−1.6 log_2-FC for *tlr3* at 6 h; −2.2 log_2-FC for *hsp70* at 48 h).

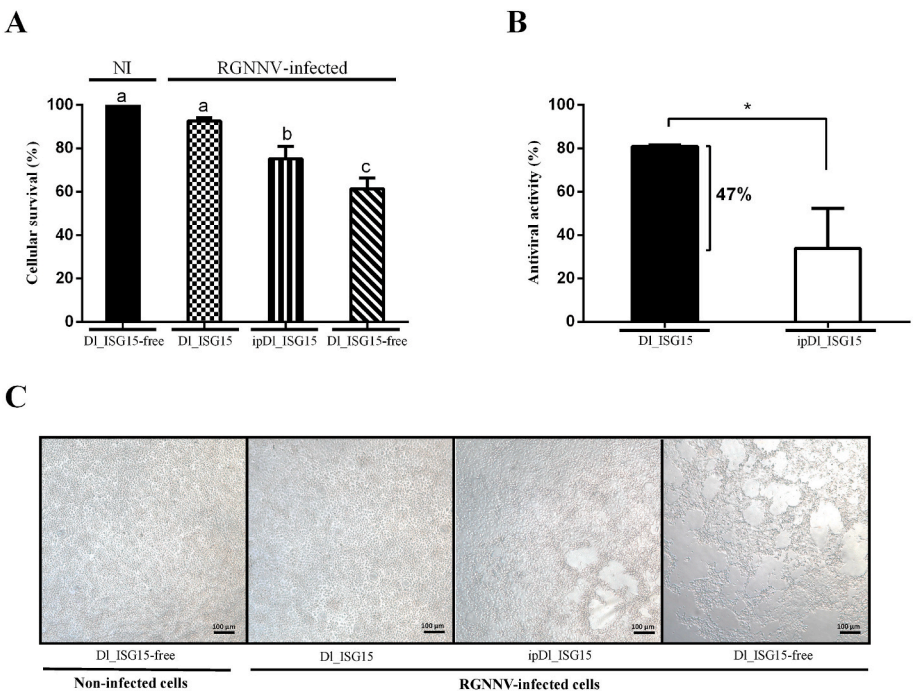

Fig. 4. Protective effect of extracellular sea bass ISG15 on RGNNV-infected E11 cells. (A) Survival rate of cells exposed to Dl_ISG15-medium (Dl_ISG15), Dl_ISG15-medium after ISG15 removal (ipDl_ISG15), or control medium. NI: non-infected. Different letters denote significant differences ($p < 0.05$). (B) Antiviral activity of Dl_ISG15-and ipDl_ISG15-medium. Asterisk denotes significant differences ($p < 0.05$). Antiviral activity was calculated as: ($A_{treatment + virus}$-$A_{no\text{-}treatment + virus}$)/($A_{no\text{-}treatment\text{-}virus}$-$A_{no\text{-}treatment + virus}$) x 100. Results are mean ± standard deviation (SD) (n = 3). (C) CPEs of cells incubated with Dl_ISG15-, ipDl_ISG15- or control-medium. Photos were taken at 6 days post-infection.

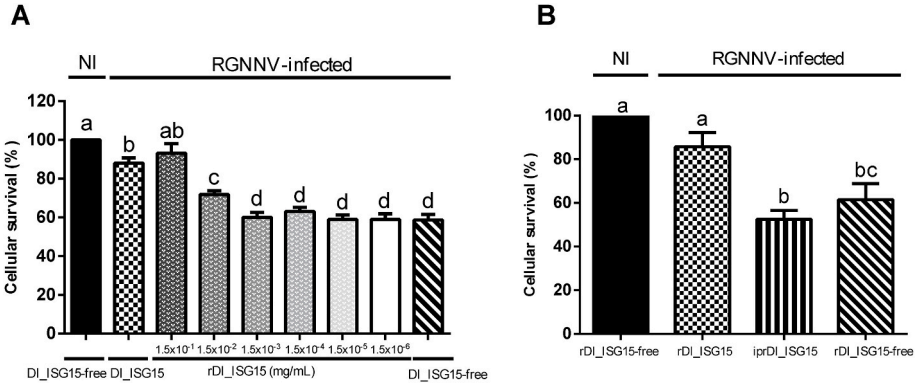

Fig. 5. Protective effect of recombinant sea bass ISG15 on RGNNV-infected E11 cells. (A) Survival rate of cells exposed to serial dilutions of rDl_ISG15. (B) Survival rate of cells exposed to rDl_ISG15 (0.15 mg/mL), rDl_ISG15 solution after ISG15 immunoprecipitation (iprDl_ISG15), or control medium. NI: non-infected. Different letters denote significant differences ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).

In order to evaluate the role of this immunomodulation in E11 protection, cells were treated with rDl_ISG15 for 48 h (maximum *mx*-induction time) prior to virus inoculation. Pre-incubation with rDl_ISG15 enhanced the cellular survival rate from 85.7% (non-pre-incubated cells) to 96.5% (pre-incubated cells) ($p < 0.05$, Fig. 6B).

The effect of ISG15 on the IFN-I system response in the course of RGNNV infections is shown in Fig. 6C. The pattern of *tlr3* and *mx* induction in infected cells exposed to ISG15 was different from that recorded in control cells. Particularly, *tlr3* was significantly induced in Dl_ISG15 group at 5 dpi (1.2 $\log_2$-FC) and 7 dpi (2.3 $\log_2$-FC) (Fig. 6C), with significant differences between both groups at both sampling times ($p < 0.05$). Regarding *mx*, it was upregulated in Dl_ISG15 group at 5 and 7 dpi (3.0 and 1.1 $\log_2$-FC, respectively), whereas the virus infection in control cells induced an earlier, although lower, *mx* upregulation (Fig. 6C). Transcription was significantly different in both groups at 2 dpi, when a downregulation was recorded in Dl_ISG15 group, as well as at 5 dpi, when the highest level of transcription in cells exposed to ISG15 was recorded.

4. Discussion

RGNNV isolates are strong inducers of European sea bass IFN-I system [16,27]. In particular, high levels of *isg15* mRNA have been reported as soon as 1 dpi in sea bass brain following the infection with the RGNNV isolate used in this work [31]. Furthermore, the anti-NNV activity of grouper (*Epinephelus coioides*) and European sea bass ISG15 proteins has been demonstrated [19,21]. These observations suggest a pivotal role of this protein in controlling NNV infections.

In the present study, the anti-RGNNV effect of extracellular Dl_ISG15 has been firstly characterised by analysing the protection conferred by serial dilutions of Dl_ISG15-medium, recording a clear dose-dependent effect. Moreover, our results indicate that Dl_ISG15 and/or other proteins that this medium may contain are present at high concentration, or are able to induce an antiviral state at low concentration, since cells incubated with the maximum dilution analysed (1:625) were still protected from RGNNV infection (Fig. 1A). In addition, the analysis of viral replication revealed a significantly lower number of viral genome copies in cells exposed to Dl_ISG15-medium than in non-exposed cells (Fig. 2), indicating that, unlike it has been reported for intracellular Dl_ISG15

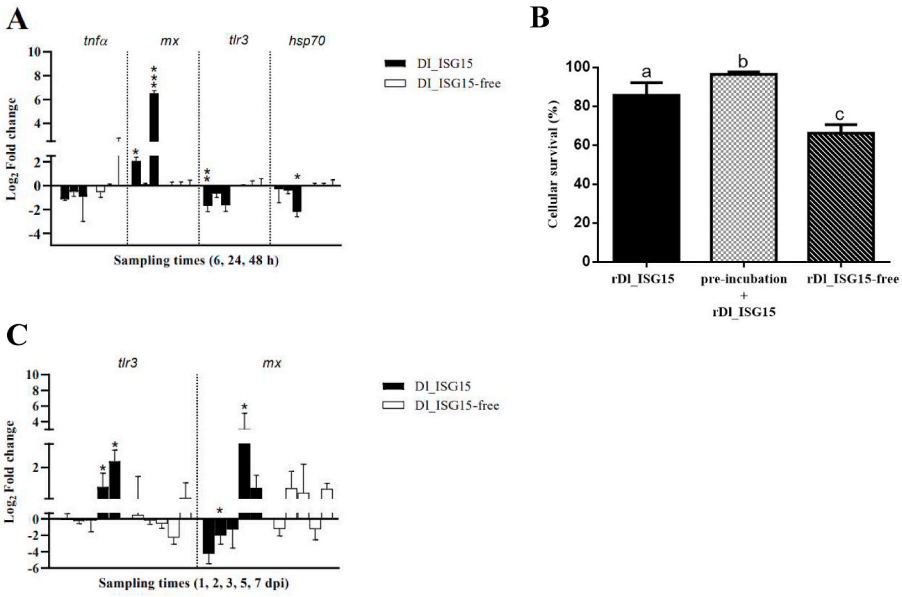

Fig. 6. Immunomodulatory effect of extracellular sea bass ISG15. (A) Relative quantification of *tnfα, mx, tlr3* and *hsp70* transcription in E11 cells incubated with Dl_ISG15-medium or control medium. Asterisks indicate significant differences between experimental groups within each sampling time (*$p < 0.05$; **< 0.01; ***< 0.001). (B) Survival rate of RGNNV-infected cells exposed to 1.5×10^{-1} mg/mL rDl_ISG15 only after viral inoculation (rDl_ISG15), or before and after viral inoculation (pre-incubation + rDl_ISG15). Non-exposed cells were used as control. Different letters denote significant differences ($p < 0.05$). (C) Relative quantification of *mx* and *tlr3* transcription in RGNNV-infected E11 cells incubated with Dl_ISG15-medium or control medium. Asterisk indicates significant differences between experimental groups within each sampling time ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).

[21], secreted sea bass ISG15 reduces RGNNV genome synthesis, which suggests different mechanisms for intracellular and extracellular sea bass ISG15, as well as a cytokine-like role for extracellular Dl_ISG15. This result has been supported by viral titration. Interestingly, the reduction in viral titre (two orders of magnitude) is more evident than the reduction of viral genome (less than one order of magnitude) at the same sampling time, suggesting a possible blocking of viral assembly and/or release in addition to viral genome replication restriction. This result reinforces the necessity of analysing both, viral genome and viral titre, in order to have a better understanding of the basis of the antiviral activity of this protein.

The cytokine-like activity of European sea bass ISG15 has also been demonstrated by incubation of RGNNV-infected cells with ipDl_ISG15-medium, which resulted in a significant decrease of the survival rate compared to cells incubated with Dl_ISG15-medium (Fig. 4). This result is in concordance with those reported by Wang et al. [18] using a similar approach. These authors demonstrated that neutralization of tongue sole ISG15 proteins with specific antibodies resulted in a significant increase of viral genome copy number, causing an enhanced viral infection. However, removal of sea bass ISG15 did not completely abolish the anti-RGNNV activity, suggesting that other cytokines secreted by Dl_ISG15-producing cells may also be involved. This cytokine-mediated protection can be considered as an indirect action of ISG15, since cytokine synthesis is probably triggered by ISG15 activity within Dl_ISG15-producing cells, as it has been demonstrated for zebrafish ISG15 expressed in EPC cells [20]. That is, ISG15 acts directly, as an extracellular cytokine that activates an antiviral response in target cells, and indirectly, by inducing the expression of cytokines within Dl_ISG15_E11 cells. According to our results, Dl_ISG15 direct action accounts for 47% of the antiviral activity reported for Dl_ISG15-medium (Fig. 4B). This percentage may depend on ISG15 concentration, which, although undetermined, it is probably low within Dl_ISG15-medium, since ISG15 visualization by western blot from cellular supernatants required a previous concentration step [21]. The identity of other cytokines involved in the protection exerted by Dl_ISG15-medium remains to be investigated. A good candidate is IFN-I system, since ISG15 has been demonstrated to regulate the transcription of IFN-I-related genes within ISG15-producing cells [20].

The direct activity of extracellular ISG15 has been clearly corroborated by exposing infected cells to different concentrations of rDl_ISG15, resulting in full protection of target cells at 1.5×10^{-1} mg/mL (Fig. 5A). Recombinant ISG15 proteins expressed in bacteria have previously been used to demonstrate their immunomodulatory effect on fish lymphocytes, showing activity at lower concentration [13,18]. The protein purification procedure used in this study could cause loss of activity, being responsible for the high ISG15 concentration required for cellular protection. ISG15 activity on target cells has been further demonstrated by rDl_ISG15 immunoprecipitation, which, unlike it has been described for ipDl_ISG15-medium, resulted in the complete abolition of cellular protection (Fig. 5B). This result demonstrates that no ISG15 molecules remain after immunoprecipitation and, therefore, supports the implication of other cytokines in the protection exerted by Dl_ISG15-medium, as suggested previously.

This is the first report of non-immune cells as targets of secreted ISG15, and demonstrates "cross-action" between different fish species, since sea bass ISG15 is acting on E11 cells, derived from snakehead fish (*Channa striata*), which may suggest that ISG15 receptor is very conserved among fish species. To our knowledge, the only previous study reporting interaction between ISG15 and non-immune cells refers to ISG15 detection on red blood cells, although the effect of that interaction is unknown [32].

Finally, the immunomodulatory activity of extracellular sea bass ISG15 has been demonstrated. Interestingly, *hsp70* and *tlr3* were downregulated in cells exposed to Dl_ISG15 (Fig. 6A). To our knowledge, this is the first report of immunogene downregulation in ISG15-treated cells.

HSP70 has been described as a pro-viral [33] or antiviral factor [34]. In fish, a pro-viral effect has been reported for viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and grass carp reovirus (CGRV) [35–37]. Whether HSP70 favours or hampers NNV infections needs further investigation. This aspect is crucial to know the impact of *hsp70* downregulation on viral infection. The induction of *hsp70* after NNV infection has been recorded *in vivo*, in Asian sea bass (*Lates calcarifer*) [38], as well as *in vitro*, in E11 and DLB-1 (derived from sea bass) cell lines [39–41]. On the contrary, *tlr3* is hardly induced in RGNNV-infected European sea bass [30], which suggests a minor role of TLR3 in the induction of IFN-I system.

Regarding *mx*, a strong induction was evidenced (Fig. 6A). The anti-NNV activity of Mx proteins has been demonstrated in Asian sea bass and grouper [42–44]. In particular, Chen et al. [45] have demonstrated interaction between grouper Mx proteins and NNV capsid proteins, which would prevent viral assembly. A similar mechanism for sea bass Mx proteins, whose synthesis is induced by Dl_ISG15, could explain the important drop in viral titre recorded in cells exposed to sea bass ISG15

(Fig. 2B). In fact, cells inoculated when *mx* transcription was maximal showed an enhanced survival rate (Fig. 6B), which suggests a key role of this protein in cellular protection.

The transcriptional analysis of *tlr3* and *mx* in RGNNV-infected cells was also addressed (Fig. 6C). Both genes were highly induced only in cells exposed to ISG15, suggesting the role of the IFN-I-dependent response in the establishment of the anti-RGNNV state recorded in this study. In fact, the restriction of RGNNV replication acting by blocking genome synthesis and hampering viral particle formation aforementioned may be consequence of the simultaneous action of different IFN-I-related proteins induced by extracellular ISG15.

In conclusion, this study contributes to a better understanding of the sea bass immune response against NNV by looking into the activity of extracellular ISG15. The direct implication of extracellular sea bass ISG15 in the protection of E11 cells from RGNNV has been demonstrated. This protection seems to involve both, reduction of viral genome replication, and interference with the generation of new infective viral particles. In addition, extracellular sea bass ISG15 protein modulates the transcription of several immuno-related genes in both, infected and uninfected cells, suggesting the cytokine-like role of this protein. This modulation probably accounts for the protection recorded in cells exposed to Dl_ISG15.

CRediT authorship contribution statement

Patricia Moreno: Formal analysis, Investigation. **Rocio Leiva-Rebollo:** Formal analysis, Investigation. **Esther Garcia-Rosado:** Supervision, Writing – review & editing. **Julia Bejar:** Supervision, Writing – review & editing. **M. Carmen Alonso:** Funding acquisition, Project administration, Supervision, Writing – original draft.

Data availability

Data will be made available on request.

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