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Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid

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ABSTRACT

In this work we have tested the *in vivo* antiviral activity of type I interferons (IFNs) in Atlantic salmon by injecting presmolts intramuscularly (i.m.) with plasmids encoding IFNa1, IFNb or IFNc under the control of a CMV promoter, and measured expression of antiviral genes in organs and protection against infection with infectious salmon anemia virus (ISAV) infection. All three IFN plasmids induced expression of antiviral genes (Mx, Viperin, ISG15 and IFIT5) at the muscle injection site while the control plasmid had little effect. Only IFNb and IFNc plasmids induced expression of antiviral genes in head kidney, liver and heart. This suggests that IFNb and IFNc are distributed systemically while IFNa1 is active only at the injection site. Injection of IFNc plasmid was found to induce expression of antiviral genes and receptors for virus RNA (RIG-I, TLR3 and TLR7) in head kidney from 1 to at least 8 weeks. Immunoblotting showed increased expression of ISG15 and Mx protein in liver with time during this time period. Challenge of presmolts with ISAV 8 weeks after injection of IFN plasmids, showed strong protection of the IFNc plasmid injected fish, low protection of the IFNb plasmid injected fish and no protection of the IFNa1 plasmid injected fish. Clues to the difference in protection obtained with IFNb and IFNc plasmids were found by immunohistochemical and immunoblot studies of Mx protein, which indicated that IFNc plasmid stimulated stronger Mx protein expression in heart tissues and liver endothelial cells than IFNb plasmid. Taken together, these data suggest that i.m. injection of the IFNc expression plasmid may be a new method for protecting Atlantic salmon against virus infection.

1. Introduction

Farmed Atlantic salmon is attacked by several viruses, which represent a continuous threat to the industry. Traditional vaccines based on inactivated virus are available for infectious pancreatic necrosis virus (IPNV), salmon pancreas disease virus (SPDV) and infectious salmon anemia virus (ISAV) and a subunit vaccine based on recombinant protein is available for IPNV [1], but these vaccines do not appear to give satisfactory protection in the farming situation. DNA vaccination provides a high level of protection against infectious hematopoietic necrosis virus (IHNV), but not other viruses [1]. This calls for improved methods for protection of farmed salmon against virus diseases.

The discovery of type I IFNs in fish opens a possibility for using them in prophylaxis against virus infections in fish. Type I IFNs are induced upon host cell recognition of viral nucleic acids [2], and protect other cells against infection by inducing numerous antiviral proteins such as Mx, ISG15, IFIT5 (ISG58) and Viperin [3–5]. In fish, four type I IFN subtypes, named IFNa, IFNb, IFNc and IFNd, have so far been characterized [6,7]. IFNa and IFNd contain 2 cysteines (2C-IFNs) while IFNb and IFNc contain 4 cysteines (4C-IFNs). The largest cluster of IFN genes has been found in Atlantic salmon, encoding two IFNa, four IFNb and five IFNc genes [6].

Atlantic salmon IFNa, IFNb and IFNc and IFNd have only 22–37% amino acid sequence identity and show major differences in cellular expression properties and antiviral activities [6,8]. IFNa1 and IFNc induced similar strong antiviral activity against IPNV and induced similar transcript levels of antiviral genes in cell lines, IFNb was less active and IFNd showed no antiviral activity [8]. IFNa1, IFNb and IFNc provided only transient inhibition of ISAV replication in TO cells [9].

In humans, pegylated recombinant IFN-α, mostly in combination with ribavirin, is used for treatment of chronic hepatitis C virus

Abbreviations: IFN, interferon; ISG, interferon-stimulated gene; TLR, Toll-like receptor; RIG-I, retinoic acid-inducible protein I; Viperin, virus inhibitory protein endoplasmatic reticulum associated interferon-inducible; ISG15, interferon-induced protein encoded by the ISG15 gene; IFIT, interferon-induced protein with tetratricopeptide repeats; Mx, myxovirus resistance; ISAV, infectious salmon anemia virus; i.m., intramuscular; i.p., intraperitoneally; RT-qPCR, reverse transcription quantitative PCR; RPS, relative percent survival.

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infections [10]. IFN-α treatment has also shown protective effects against influenza virus infection in mammals and chicken [11–13]. However, IFN prophylaxis to combat virus diseases in domestic animals and human has apparently had limited success due to the costs of recombinant IFNs, their rapid degradation in the body and side effects. Reports on effects of IFNs against virus infection in live fish are scarce. Treatment of rainbow trout with recombinant Atlantic salmon IFNa2 injected intraperitoneally (i.p.) provided protection against IHNV infection for up to 7 days, which is not enough for prophylaxis of farmed fish [14]. In the present work we have used a more novel approach by studying antiviral effects of intramuscular (i.m.) injection of IFN expressing plasmids in Atlantic salmon. The results showed surprising differences among IFNa, IFNb and IFNc plasmids in their ability to induce systemic expression of antiviral genes and to protect salmon from infection with a high virulent strain of ISAV. Notably, i.m. injection of IFNc plasmid provided systemic up-regulation of antiviral genes in salmon for at least 8 weeks accompanied by a high level of protection against ISAV infection.

2. Materials and methods

2.1. Fish

Atlantic salmon (*Salmo salar* L.) presmolts (35–45 g) of the strain Aquagen standard (Aquagen, Kyrksæterøra, Norway) were kept at Tromsø Aquaculture Research Station, Norway in 300 l tanks supplied with fresh water at 10 °C and were fed commercial dry food. Prior to treatments, the fish were anesthetized with 0.005% benzocaine (ACD Pharmaceuticals, Norway). Fish groups were labeled by tattooing (2% alcian blue, Panjet inoculator). The fish were killed by an overdose benzocaine prior to harvest of organs. All handling of fish was in accordance with the Norwegian "Regulation on Animal Experimentation" and all fish experiments were submitted to and approved by the Norwegian Animal Research Authority (NARA) before initiation.

2.2. Plasmids used for intramuscular injection

Interferon plasmids encoding the open reading frame (ORF) of Atlantic salmon IFNa1, IFNb and IFNc were available from a previous study [15]. All the three IFN ORFs were sub-cloned into the pcDNA3.3-TOPO vector (Invitrogen) downstream of the CMV promoter. A religated pcDNA3.3 plasmid without insert was used as negative control. Plasmids were transformed and grown in One Shot TOP10 *Escherichia coli* (Invitrogen) and purified by EndoFree plasmid purification kit (Qiagen).

2.3. Antibodies

Polyclonal antibodies against Atlantic salmon Mx and ISG15 proteins were as described [16,17].

2.4. Fish experiments for RT-qPCR, immunoblotting and immunohistochemistry

Three experiments were performed where five groups of presmolts kept in one tank were injected intramuscularly (i.m.) approximately 1 cm below the dorsal fin with 15 μg plasmid in 50 μl sterile phosphate-buffered saline (PBS) at pH 7.4 or with PBS only. In Experiments 1–3, fish groups were injected with IFNa1, IFNb or IFNc plasmid or control plasmid. In Experiment 4, fish groups were injected with IFNc, control plasmid or PBS. Muscle tissue at the injection site and organs were harvested at different time intervals after injection and stored in RNA*later* (Ambion) for RNA extraction or stored in liquid nitrogen for protein extraction. Experiment 1 (Fig. 1): muscle, head kidney and liver were harvested 7 days post-injection (dpi) for RT-qPCR ($n = 5$). Experiment 2 (Figs. 5A, B and 6): at 56 dpi, livers were harvested for immunoblotting ($n = 3$) and liver and heart were harvested for immunohistochemistry ($n = 4$). Experiment 3 (Fig. 5C): at 14 dpi heart tissues were harvested for immunoblotting ($n = 4$). Experiment 4: organs were sampled at 5, 7, 14, 21, 35 and 56 dpi. Muscle and head kidney were sampled ($n = 5$) at all time points for RT-qPCR (Fig. 2A, B and C). Muscle, liver, spleen, gut, heart and gill were harvested ($n = 5$) for RT-qPCR at 7 dpi (Supplementary Fig. 2). Livers were harvested ($n = 4$) for immunoblotting at 7, 21 and 56 dpi (Fig. 3).

2.5. Challenge experiment with ISAV

Groups of presmolts (50 fish per group) kept in one tank were injected i.m. with IFN plasmids, control plasmid or PBS as described in 2.3. Eight weeks after injection each fish was injected i.p. with 100 μl L-15 medium containing 10^4 $TCID_{50}$ units of the ISAV Glesvaer/2/90 strain [9]. Mortality was recorded every day and 28 days post-virus injection relative percentage survival (RPS) in the groups was calculated as [1 − (% mortality in test group/% mortality in control plasmid group)] × 100.

2.6. Reverse transcription quantitative PCR (RT-qPCR)

Organ samples or leukocytes were collected in RLT buffer and RNA was isolated with the RNeasy Mini kit (Qiagen). One microgram RNA was subjected to cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen). Transcripts of IFNs, Mx, ISG15, Viperin, IFIT5 (also named ISG58), RIG-I, TLR7, TLR3 in cDNA from organs or leucocytes were analyzed by qPCR using 7500 Fast Real-Time PCR System (Applied Biosystems) as described previously [15]. Relative quantifications of gene transcripts were performed by the Pfaffl method [18], using Elongation Factor 1αB (EF1αB) as reference gene [19].

2.7. Detection of Mx and ISG15 protein expression by immunoblotting

Frozen organs were weighed and transferred to 2 ml microtubes and tissue lysis buffer (Tissue Extraction Reagent I, Invitrogen) was added (100 mg tissue in 100 μl lysis buffer). Homogenization was performed with Precellys beads and homogenizer (Precellys®24, Bertin Technologies) at 5900 rpm for 20 s. After centrifugation for 5 min at 10,000 × *g* at 4 °C, protein concentration in the supernatants was measured with BCA protein assay kit (Pierce, Thermo Science). Supernatants (10 μg protein per well) were subjected to LDS-electrophoresis on a 4–12% NuPAGE Bis-Tris Gel (Invitrogen). Blotting, antibody incubations and development of blots were done as described previously [9].

2.8. Detection of Mx protein expression by immunohistochemistry

Organs were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C and embedded in paraffin wax by routine procedures. Tissue sections (4 μm) were cut and mounted onto poly-L-lysine coated slides, dried and cleared with HistoClear solution (National Diagnostics). After rehydration, slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 30 min followed by incubation in 1% hydrogen peroxide for 15 min. The slides were blocked with 5% nonfat dried milk powder (AppliChem) for 2 h and subsequently incubated with anti-Mx antibody (1:500) for 16 h at 4 °C and with HRP-conjugated antibody (1:2000, goat anti-rabbit IgG, Invitrogen) for 1 h. Red color showing Mx staining was developed by incubation with 100 μl AEC Substrate Chromogen (Dako) for 10 min and the sections were then counterstained with Mayer's hematoxylin (Sigma).

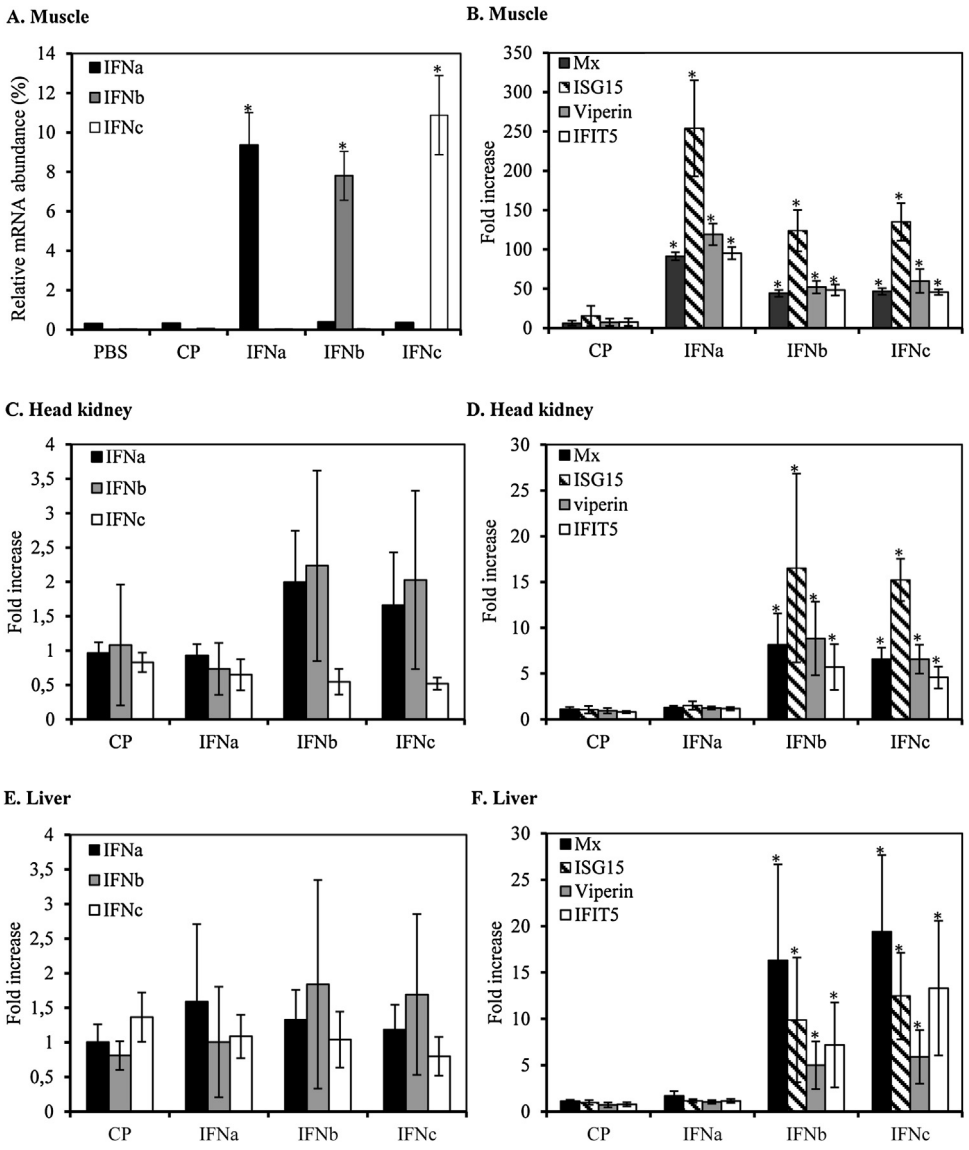

Fig. 1. Induction of antiviral genes in salmon presmolts after i.m. injection of IFN expression plasmids. Five groups of presmolts were injected i.m. with PBS, control plasmid (CP) or plasmids expressing IFNa1, IFNb or IFNc, respectively ($n = 5$). Expression of IFNa1, IFNb, IFNc, Mx, Viperin, ISG15 and IFIT5 was measured by RT-qPCR 7 days after injection. A and B, muscle tissue at the plasmid injection site. C and D, head kidney. E and F, liver. Transcription of IFNa, IFNb and IFNc in muscle is presented as relative mRNA abundance compared to the Elongation Factor 1αB reference gene, while other values are presented as fold increase in transcripts (mean ± SD) compared to PBS injected fish. Statistical significant differences ($p < 0.05$) between IFN plasmid groups and control plasmid group are indicated with a star (*).

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism vision 6.01 for Windows. Gene transcripts in organs or leukocytes were compared using an unpaired Student's *t*-test and considered as statistically significant at $p \leq 0.05$. The differences in mortality and survival rate were compared using chi square test and considered as statistically significant at $p \leq 0.01$.

3. Results

3.1. Expression of antiviral genes in organs after intramuscular (i.m.) injection with IFN expression plasmids

As expected i.m. injection of expression plasmids for IFNa1, IFNb and IFNc into Atlantic salmon presmolts resulted in strong expression of the respective IFNs in the muscle tissue (Fig. 1A). Consequently, all three IFN plasmids caused strong induction of the antiviral genes Mx, Viperin, ISG15 and IFIT5 at the muscle injection site (Fig. 1B). This is most likely due to release of IFN from muscle cells that have taken up plasmid, since transfection of the IFN expression plasmids into HEK293 cells resulted in secretion of functional IFNs [8]. IFNa1 plasmid seemed to have a somewhat stronger effect compared to the IFNb and IFNc plasmids, which had similar effects.

Interestingly, i.m. injections of both IFNb and IFNc plasmids also caused up-regulation of the antiviral genes in head kidney (Fig. 1D) and liver (Fig. 1F) whilst neither IFNa1 nor control plasmid had any effect. Similar results have been observed in four independent fish experiments. Injections of IFNb and IFNc plasmids caused a minor up-regulation of IFNa and IFNb in head kidney while IFNc expression was unchanged (Fig. 1C). None of the IFNs were up-regulated in liver by injections of the IFN-plasmids (Fig. 1E). Taken together, this suggests that i.m. injection of IFNb and IFNc plasmids cause systemic up-regulation of antiviral genes due to release of IFNs at the muscle injection site while IFNa1 plasmid only up-regulates ISGs at the injection site. Mx expression was compared in several organs of fish 7 days after injection of IFNc plasmid, which showed

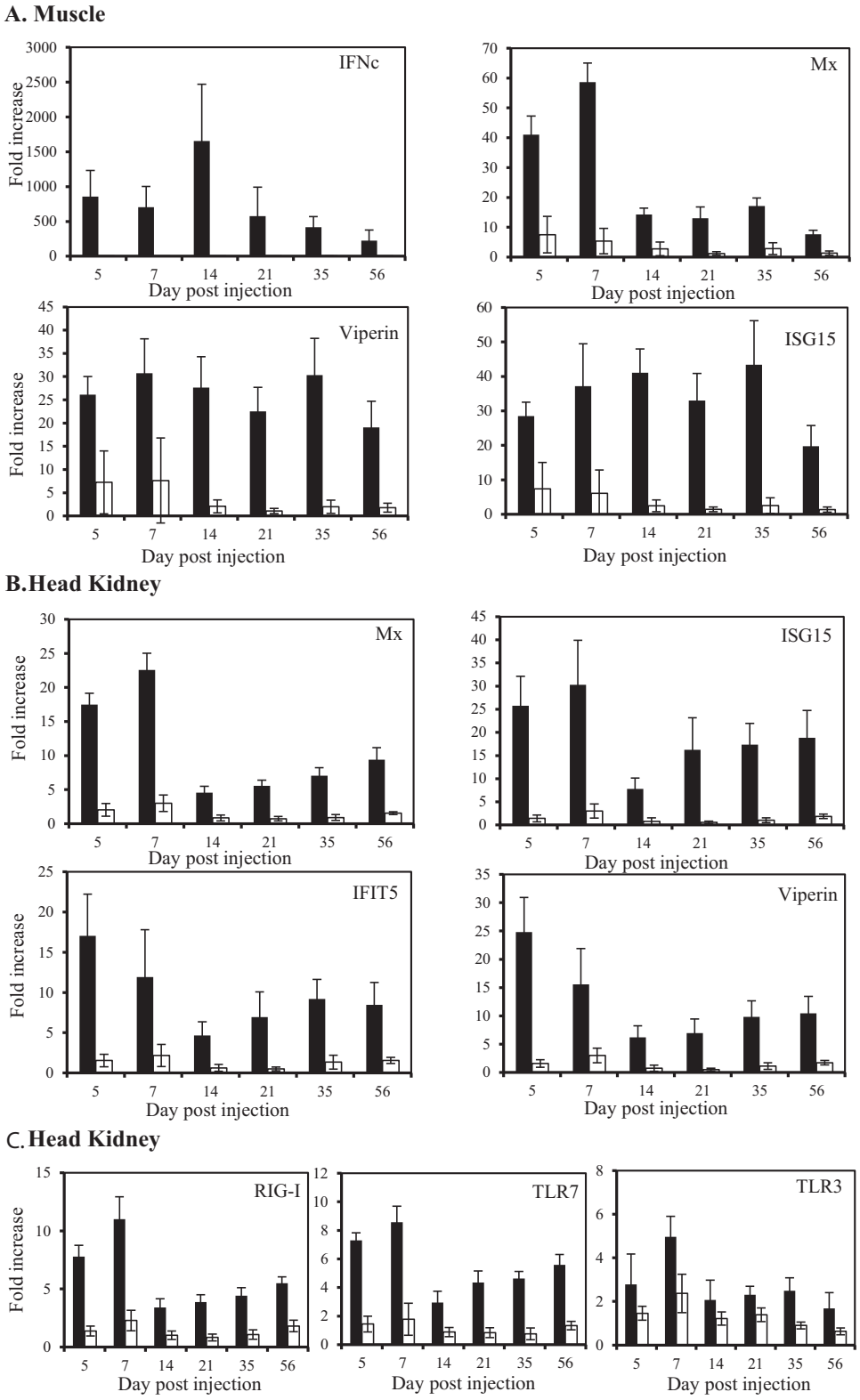

Fig. 2. Time course study of IFNc and ISGs transcript levels in fish injected with IFNc plasmid, control plasmid or PBS. (A) Expression of IFNc, Mx, Viperin and ISG15 in muscle tissue at the plasmid injection site. (B) Expression of antiviral genes in head kidney. (C) Expression of viral RNA receptors in head kidney. Gene expression was measured by RT-qPCR at the indicated days post-injection. Values are fold increase in transcripts compared to PBS injected fish ($n = 5$). Black bars: IFNc plasmid group, white bars: control plasmid group.

highest increase in liver followed by heart, head kidney, spleen, gut and gills (Suppl. Fig. 1).

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.05.059.

3.2. *Effect of IFNa1 and IFNc on expression of ISGs in head kidney leucocytes*

Since the IFNc plasmid, but not the IFNa1 plasmid induced expression of ISGs in head kidney, we wanted to study if

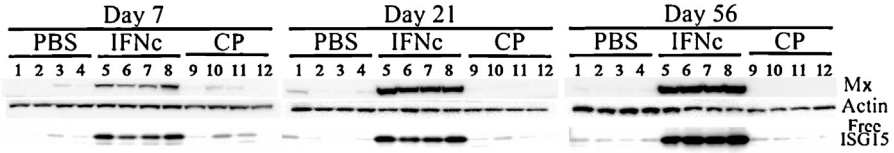

Fig. 3. Time course study of Mx and ISG15 protein expression in liver of presmolts injected i.m. with IFNc plasmid, control plasmid or PBS. Liver tissues were harvested at 7, 21 and 56 days after injection and analyzed for Mx and ISG15 expression by immunoblotting. Gels were loaded with 10 μg protein from each of four fish from PBS group (lanes 1–4), IFNc plasmid group (lanes 5–8) and control plasmid group (CP, lanes 9–12). Blots were incubated with anti-Mx and anti-actin antibody or anti-ISG15 and anti-actin antibody and are representative of two independent experiments.

recombinant IFNa1 and IFNc might have different effects on induction of ISGs in head kidney leucocytes. However, recombinant IFNa1 and IFNc up-regulated the antiviral genes Mx, ISG15, Viperin and IFIT5 (ISG58) to similar extents in head kidney leucocytes (Suppl. Fig. 2A). Moreover, IFNa1 and IFNc also up-regulated similarly the viral RNA receptors RIG-I, TLR3 and TLR7, which activate IFN transcription upon binding of virus RNA (Suppl. Fig. 2B). Lack of systemic induction of ISGs by IFNa1 plasmid is thus not likely to be due to lack of response to IFNa1 in organs.

Supplementary Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.05.059.

3.3. Time course study of antiviral gene expression in muscle and head kidney after injection of IFNc plasmid

To study if i.m. injection of IFNc plasmid had a prolonged effect on expression of antiviral genes in salmon, groups of presmolts were i.m. injected with IFNc plasmid, control plasmid or PBS and measured for gene transcripts in muscle at the injection site and head kidney for a period of 5–56 days. IFNc, Mx, Viperin and ISG15 expression were increased in muscle of IFNc plasmid injected fish throughout the experimental period (Fig. 2A). IFNc showed highest expression in muscle at day 14 after injection and a declining expression in the follow sampling days. Mx expression in muscle of IFNc plasmid injected fish was highest at day 7 and then declined while ISG15 was elevated through day 35 and declined at day 56. Mx expression in head kidney was highest at day 7, declined to a low level at day 14 and then gradually increased (Fig. 2B). A similar trend of expression in head kidney was found for ISG15, IFIT5 and Viperin, and the virus RNA receptors RIG-I, TLR3 and TLR7 (Fig. 2C).

3.4. Time course study of Mx protein expression in liver after injection of IFNc plasmid

Since we observed increased ISG levels in head kidney throughout the 56 days after injection of IFNc plasmid, we wanted to study ISG protein levels in internal organs. For this purpose, we performed immunoblotting of Mx and ISG15 proteins in liver at 7, 21 and 56 days after i.m. injection of IFNc plasmid, control plasmid and PBS. As shown in Fig. 3, Mx protein was hardly detected in liver from control plasmid and PBS injected fish at any time point. In contrast, Mx protein was detected in liver of all 4 individuals 7 days after injection of IFNc plasmid and increased at day 21 and 56. A similar increase in expression pattern was observed for ISG15 (Fig. 3).

3.5. Effect of IFN plasmids on protection of salmon against infection by ISAV

Since injection of IFNb and IFNc plasmid induced antiviral genes systemically in Atlantic salmon, we wanted to find out if the IFN plasmids might provide protection of salmon against virus infection. For this purpose we chose to challenge the fish with a high virulent strain of the orthomyxovirus ISAV, which is known to cause a high level of mortality in salmon in challenge experiments [20]. Groups of presmolts were injected i.m. with IFNa1 plasmid, IFNb plasmid, IFNc plasmid, control plasmid or PBS and kept in a fresh water tank for 8 weeks before injection with 10^4 $TCID_{50}$ Units of ISAV4. Mortality started to develop at day 16 post-infection and reached 82% and 91% in the PBS and control plasmid groups, respectively, at day 28 when the experiment was terminated (Fig. 4). The mortality in the IFNa1 plasmid injected fish developed at a similar rate as in the control groups and reached 86% while the mortality in the IFNb plasmid injected fish developed somewhat slower and reached 75%, which gives a relative percent survival (RPS) of 5.5% (IFNa) and 17.6% (IFNb) ($p > 0.05$). In contrast to the other groups, the IFNc group did not show mortality until day 26 and reached a total mortality of only 6% at the end of the experiment, which gives a RPS of 93.4% ($p < 0.01$). Similar results were obtained in another challenge experiment. These experiments thus confirmed the antiviral gene results, which suggested that injection of IFNa1 plasmid does not provide systemic protection of salmon against virus infection while injection of IFNc plasmid gives a high level of protection even after 8 weeks. Surprisingly, however, the IFNb plasmid only provided a low level of protection despite the fact that it also caused systemic induction of antiviral genes.

3.6. Comparison of antiviral protein expression in liver and heart after injection of IFN plasmids

As the IFN plasmids showed such a large difference in protective effect 8 weeks after injection, we wanted to study if they induced different levels of antiviral proteins in liver and heart, which are strongly affected by ISAV infection. Immunoblotting of Mx and ISG15 were used for this purpose. As shown in Fig. 5A and B, fish injected with IFNb and IFNc plasmids showed similar strong

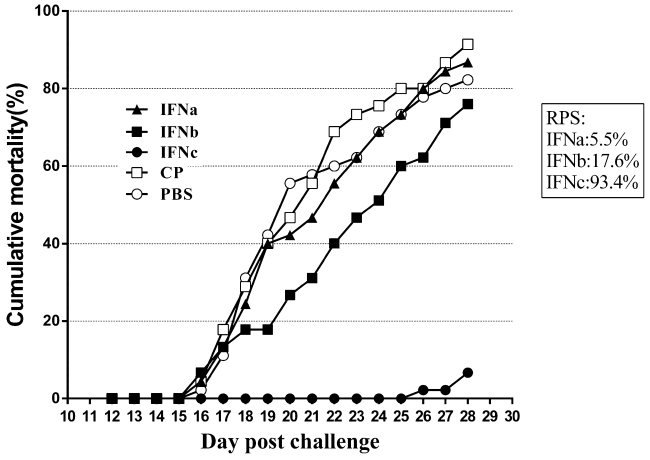

Fig. 4. Effect of IFN plasmids on protection of salmon against infection by ISAV. Groups of presmolts ($n = 50$) were injected i.m. with IFNa1 plasmid, IFNb plasmid, IFNc plasmid, control plasmid or PBS. Eight weeks after injection the fish were injected i.p. with 10^4 $TCID_{50}$ units of ISAV. Mortality in the groups was recorded each day from 1 to 28 days after injection and RPS was calculated from the cumulative mortality at day 28.

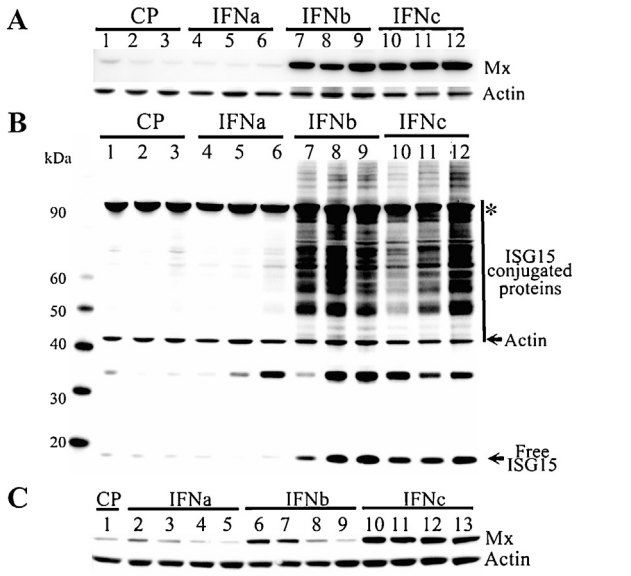

Fig. 5. Immunoblotting of Mx and ISG15 protein in liver and heart of presmolts after i.m. injection of IFNa1, IFNb, IFNc or control plasmid. Liver tissues were harvested at day 56 after injection and analyzed for Mx (A) and ISG15 (B). Lanes 1–3: control plasmid (CP). Lanes 4–6: IFNa1 plasmid. Lanes 7–9: IFNb plasmid. Lanes 10–12: IFNc plasmid. Non-specific binding with the anti-ISG15 antibody was observed with a 90 kDa protein band (indicated with *) in all fish samples. (C) Heart tissues were harvested at 14 days after injection and analyzed for Mx expression. Lane 1: Control plasmid (CP). Lanes 2–5: IFNa1 plasmid. Lanes 6–9: IFNb plasmid. Lanes 10–13: IFNc plasmid. Each lane was loaded with 10 μg protein from one individual except lane 1 in (C), which represents pooled protein extracts from four fish injected with control plasmid. Actin was used as a control for equal loading of protein.

expression of Mx, free ISG15 or ISG15 conjugates in liver 8 weeks after injection while fish injected with IFNa1 plasmid or control plasmid showed faint or no expression of these proteins. These data did thus not resolve the difference in protection obtained with the IFNb and IFNc plasmids. However, IFNc plasmid induced a higher level of Mx protein in heart compared to IFNb plasmid although this experiment was conducted 14 days after plasmid injection (Fig. 5C). Mx protein was at similar low levels in heart of fish injected with IFNa1 and control plasmid.

3.7. Immunohistochemistry of Mx protein in heart and liver from fish injected with IFNa1, IFNb and IFNc plasmids

The difference in protective effects between IFNb and IFNc plasmids might be due to differences in induction of antiviral proteins in cell types, which are important for ISAV infectivity. Accordingly, we decided to do immunohistochemistry of Mx protein in liver and heart of fish 8 weeks after injection with PBS or IFNa1, IFNb and IFNc plasmids (Fig. 6). Mx-staining was observed throughout the liver tissue from IFNb and IFNc treated fish (Fig. 6C and D) while little Mx-staining was seen in liver of PBS and IFNa1 treated fish (Fig. 6A and B). In the IFNb and IFNc groups, Mx was relatively strongly stained in some cells resembling mammalian Kuppfer cells and more weakly stained in hepatocytes. Interestingly, endothelial cells of blood vessels appeared to be more strongly stained for Mx in liver from fish treated with IFNc plasmid than from fish treated with IFNb plasmid. In heart, stratum compactum and stratum spongiosum was strongly stained in IFNc plasmid treated fish (Fig. 6H), but more weakly stained in fish treated with IFNb plasmid (Fig. 6G). Heart from fish treated with PBS or IFNa1 plasmid showed little or no staining (Fig. 6E and F).

4. Discussion

Previous work has shown that recombinant IFNa1, IFNb and IFNc protect salmon cells against IPNV and ISAV infection *in vitro*, IFNa1 and IFNc having similar and stronger antiviral activity than IFNb [8,9]. In the present work we have studied *in vivo* antiviral activity of these IFNs delivered as genes in expression plasmids injected i.m., which demonstrated that IFNb and IFNc plasmids, but not IFNa1 plasmid induced systemic up-regulation of antiviral genes in live Atlantic salmon. Notably, only i.m. injection of IFNc plasmid provided a high level of protection of Atlantic salmon against infection by a high virulent strain of ISAV for at least 8 weeks after injection.

The fact that all three IFN expression plasmids induced similar levels of ISG transcripts at the muscle injection site, suggests that similar amounts of IFNa1, IFNc and IFNb were produced by the muscle cells. In contrast, only IFNb and IFNc plasmids induced antiviral genes in head kidney, liver and heart. The lack of induction of antiviral genes by IFNa1 plasmid injection is not due to lack of effect of IFNa1 on head kidney cells, since recombinant IFNa1 and IFNc induced similar levels of ISG transcripts in head kidney leucocytes. These results thus suggest that IFNc and IFNb are distributed through the circulation and induce antiviral genes systemically in the fish while IFNa is only active at the production site. During a virus infection, IFNa is thus probably mainly important at the virus infection site while IFNc and IFNb may be distributed systemically and trigger synthesis of antiviral proteins in cells throughout the fish body. In this context IFNc appears to be a main player in innate antiviral responses of Atlantic salmon since it is produced by a variety of cell types, is induced by both viral dsRNA and ssRNA analogs and has equally strong antiviral activity as IFNa1 [8]. While IFNb is also distributed systemically, it has less antiviral activity than IFNa and IFNc, is produced mainly by specialized leukocytes and was mainly induced by the ssRNA analog [8]. The difference in distribution properties of IFNa compared to IFNb and IFNc may have several explanations. The number of disulphide bridges might possibly influence the degradation rate of the IFNs. IFNa is a 2C-IFN, which contains one disulphide bridge, while IFNb and IFNc are 4C-IFNs, which contain two disulphide bridges [21]. However, the isoelectric points of IFNa1 (p*I* 9.2) and IFNb/IFNc (p*I* 6.9/p*I* 5.1) are also quite different and might influence their distribution and degradation properties.

The time course study showed that IFNc plasmid induced up-regulation of not only antiviral genes (Mx, ISG15, Viperin, IFIT5), but also genes for receptors of virus RNA (RIG-I, TLR3 and TLR7) in head kidney throughout the 8 week experimental period. This suggests that fish injected with IFNc plasmid indeed possess increased innate immunity to virus infection compared to fish injected with IFNa1 or control plasmid. Increased expression of Mx and ISG15 protein was confirmed both in liver and heart of IFNc plasmid injected fish 8 weeks after injection. It is thus highly likely that injected IFNc plasmid may continue to provide systemic expression of antiviral genes beyond the 8 weeks experimental period. This finding inspired us to investigate if injection of IFNc plasmid might in fact provide protection of Atlantic salmon against virus infection even at 8 weeks after plasmid injection. For this purpose we chose a high virulent strain of ISAV, which is an orthomyxovirus that causes high mortality in Atlantic salmon presmolts. The challenge experiment indeed showed that IFNc provided a high level of protection against ISAV induced mortality in salmon 8 weeks after plasmid injection while IFNa1 and control plasmid provided no protection compared to PBS injected fish. Surprisingly, injection of IFNb plasmid gave a low level of protection against ISAV infection despite the fact that IFNb and IFNc plasmids induced comparable amounts of Mx and ISG15 protein in liver 8 weeks after injection. This may be due to that IFNb and IFNc use different receptors and consequently induce antiviral proteins in different cell types. This

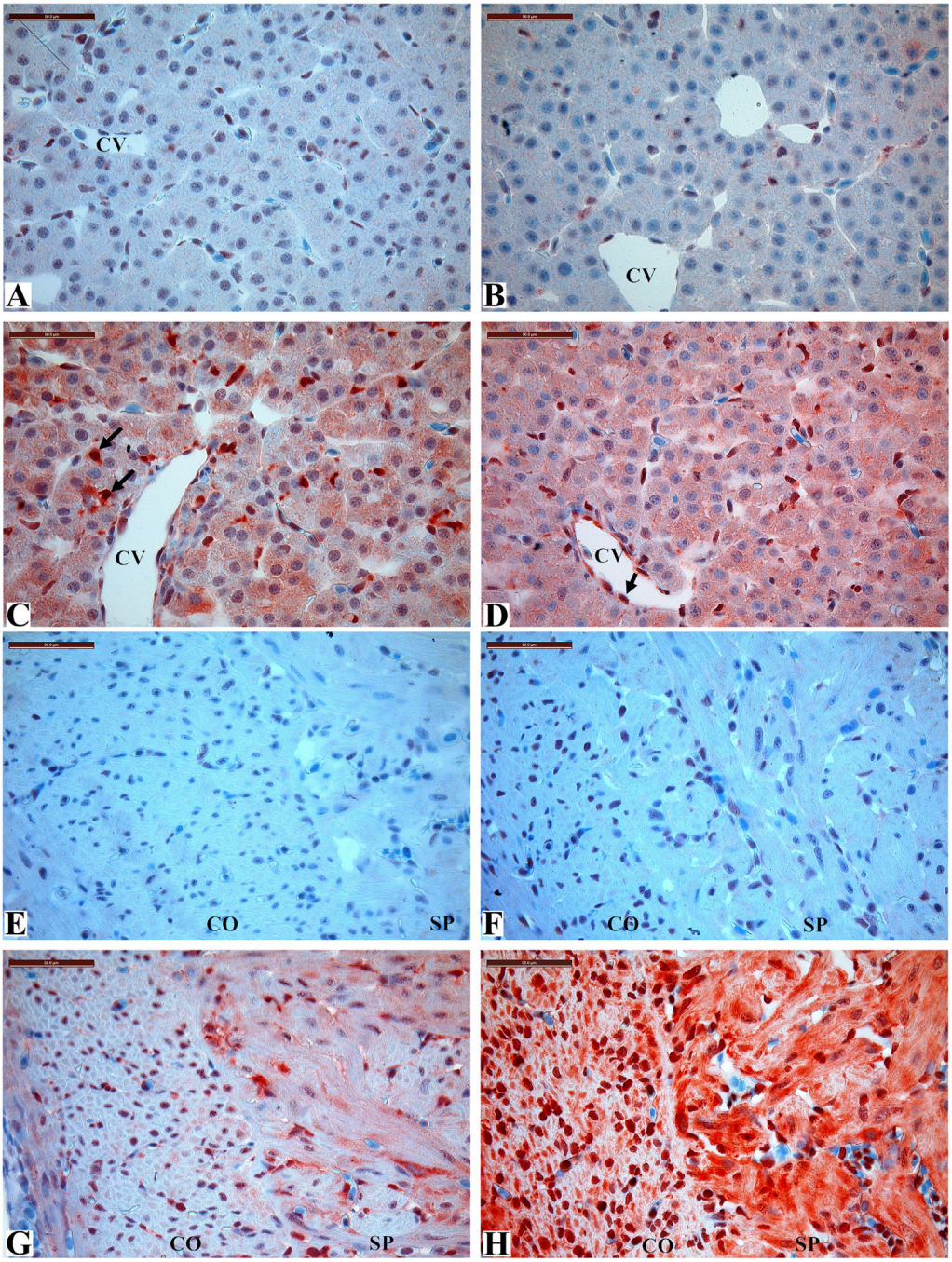

Fig. 6. Immunohistochemistry of Mx protein in liver and heart 8 weeks after i.m. injection of IFN plasmids. Liver sections from fish injected with control plasmid (A), IFNa1 plasmid (B), IFNb plasmid (C) and IFNc plasmid (D). Heart sections from fish injected with control plasmid (E), IFNa1 plasmid (F), IFNb plasmid (G) and IFNc plasmid (H). Red color shows staining for Mx protein. Arrows indicate macrophage like cells in (C) and endothelial cells in (D), which show strong staining for Mx protein.

idea was examined by immunohistochemistry of Mx protein in heart and liver, which are strongly affected by ISAV infection. Focal necrosis in liver of ISAV infected fish is commonly found, but the main target cells for infection by ISAV are endothelial cells lining the circulatory system and not hepatocytes [22]. Sections of liver from IFNb and IFNc treated fish showed similar Mx-staining except that endothelial cells appeared to be more strongly stained in IFNc treated fish compared to IFNb treated fish. This may thus in part explain the differences in protection obtained with IFNc compared to IFNb plasmid. Moreover, heart tissue showed stronger Mx staining throughout in fish treated with IFNc plasmid compared to IFNb plasmid, which was confirmed by immunoblotting of Mx. This suggests that IFNc induces antiviral proteins more strongly than IFNb in several different cell types in heart. Other explanations may, however, also be possible since mammalian type I IFNs are known to have a wide range of biological activities such as sensitizing cells to apoptosis upon subsequent viral infection [23], stimulation of cytotoxic activity of NK cells [24] and stimulation of cells involved in adaptive immune responses [25]. The difference in effect of IFNb and IFNc may be due to differences in use of receptors, which is currently under investigation by our group.

Whether i.m. injection of IFNc plasmid might be a usable method for combating virus infections in farmed salmon depends on several questions, which have to be answered in future studies. Among those are the duration of the antiviral effects of IFNc plasmid injection, whether IFNc plasmid protects against other viruses

and eventual side effects. For example, it needs to be examined if IFNc plasmid injection affects the general performance of the fish such as growth and smoltification. In such studies the level of IFNc expression may be controlled by the plasmid dose and/or by using promoters other than the CMV promoter. The benefit of using IFNc plasmid in prophylaxis against virus infections is that it induces antiviral genes with a broad spectrum of antiviral properties while conventional DNA vaccines are designed to induce adaptive immune responses that are directed toward specific pathogens.

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References

[1] Gomez-Casado E, Estepa A, Coll JM. A comparative review on European-farmed finfish RNA viruses and their vaccines. Vaccine 2011;29:2657–71.
[2] Takeuchi O, Akira S. Recognition of viruses by innate immunity. Immunol Rev 2007;220:214–24.
[3] Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. Nat Rev Immunol 2008;8:559–68.
[4] Abbas YM, Pichlmair A, Gorna MW, Superti-Furga G, Nagar B. Structural basis for viral 5′-PPP-RNA recognition by human IFIT proteins. Nature 2013;494:60–4.
[5] Chin KC, Cresswell P. Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc Natl Acad Sci U S A 2001;98:15125–30.
[6] Sun B, Robertsen B, Wang Z, Liu B. Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. Dev Comp Immunol 2009;33:547–58.
[7] Chang M, Nie P, Collet B, Secombes CJ, Zou J. Identification of an additional two-cysteine containing type I interferon in rainbow trout *Oncorhynchus mykiss* provides evidence of a major gene duplication event within this gene family in teleosts. Immunogenetics 2009;61:315–25.
[8] Svingerud T, Solstad T, Sun B, Nyrud ML, Kileng O, Greiner-Tollersrud L, et al. Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc-producing cells in fish lymphoid tissues. J Immunol 2012;189:5912–23.
[9] Svingerud T, Holand JK, Robertsen B. Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture. Virus Res 2013;177:163–70.
[10] George PM, Badiger R, Alazawi W, Foster GR, Mitchell JA. Pharmacology and therapeutic potential of interferons. Pharmacol Ther 2012;135:44–53.
[11] Meng S, Yang L, Xu C, Qin Z, Xu H, Wang Y, et al. Recombinant chicken interferon-alpha inhibits H9N2 avian influenza virus replication in vivo by oral administration. J Interferon Cytokine Res 2011;31:533–8.
[12] Van Hoeven N, Belser JA, Szretter KJ, Zeng H, Staeheli P, Swayne DE, et al. Pathogenesis of 1918 pandemic and H5N1 influenza virus infections in a guinea pig model: antiviral potential of exogenous alpha interferon to reduce virus shedding. J Virol 2009;83:2851–61.
[13] Kugel D, Kochs G, Obojes K, Roth J, Kobinger GP, Kobasa D, et al. Intranasal administration of alpha interferon reduces seasonal influenza A virus morbidity in ferrets. J Virol 2009;83:3843–51.
[14] Ooi EL, Verjan N, Haraguchi I, Oshima T, Kondo H, Hirono I, et al. Innate immunomodulation with recombinant interferon-alpha enhances resistance of rainbow trout (*Oncorhynchus mykiss*) to infectious hematopoietic necrosis virus. Dev Comp Immunol 2008;32:1211–20.
[15] Svingerud T, Solstad T, Sun BJ, Nyrud MLJ, Kileng O, Greiner-Tollersrud L, et al. Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc-producing cells in fish lymphoid tissues. J Immunol 2012;189:5912–23.
[16] Sun B, Skjaeveland I, Svingerud T, Zou J, Jorgensen J, Robertsen B. Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. J Virol 2011;85:9188–98.
[17] Rokenes TP, Larsen R, Robertsen B. Atlantic salmon ISG15: expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. Mol Immunol 2007;44:950–9.
[18] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 2001;29:e45.
[19] Lauscher A, Krossoy B, Frost P, Grove S, Konig M, Bohlin J, et al. Immune responses in Atlantic salmon (*Salmo salar*) following protective vaccination against infectious salmon anemia (ISA) and subsequent ISA virus infection. Vaccine 2011;29:6392–401.
[20] Mjaaland S, Markussen T, Sindre H, Kjoglum S, Dannevig BH, Larsen S, et al. Susceptibility and immune responses following experimental infection of MHC compatible Atlantic salmon (*Salmo salar* L.) with different infectious salmon anaemia virus isolates. Arch Virol 2005;150:2195–216.
[21] Hamming OJ, Lutfalla G, Levraud JP, Hartmann R. Crystal structure of Zebrafish interferons I and II reveals conservation of type I interferon structure in vertebrates. J Virol 2011;85:8181–7.
[22] Aamelfot M, Dale OB, Weli SC, Koppang EO, Falk K. Expression of the infectious salmon anemia virus receptor on Atlantic salmon endothelial cells correlates with the cell tropism of the virus. J Virol 2012;86:10571–8.
[23] Tanaka N, Sato M, Lamphier MS, Nozawa H, Oda E, Noguchi S, et al. Type I interferons are essential mediators of apoptotic death in virally infected cells. Genes Cells 1998;3:29–37.
[24] Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189–220.
[25] Tovey MG, Lallemand C, Thyphronitis G. Adjuvant activity of type I interferons. Biol Chem 2008;389:541–5.