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Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model

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ABSTRACT

There is a need for more efficient vaccines to combat viral diseases of Atlantic salmon and other farmed fish. DNA vaccines are highly effective against salmonid rhabdoviruses, but have shown less effect against other viruses. In the present work we have studied if type I IFNs might be used as adjuvants in fish DNA vaccines. For this purpose we chose a DNA vaccine model based on the hemagglutinin-esterase (HE) gene of infectious salmon anemia virus (ISAV) as antigen. Salmon presmolts were injected with a plasmid encoding HE alone or together with a plasmid encoding Atlantic salmon type I IFN (IFNa1, IFNb or IFNc). Sera were harvested after 7–10 weeks for measurements of antibody against ISAV and the fish were challenged with ISAV to measure protective effects of the vaccines. The results showed that all three IFN plasmids delivered together with HE plasmid potently enhanced protection of salmon against ISAV mediated mortality and stimulated an increase in IgM antibodies against the virus. In contrast, HE plasmid alone gave low antibody titers and a minor protection against ISAV. This demonstrates that type I IFNs stimulate adaptive immune responses in fish, which may be a benefit also in other fish DNA vaccines. Quantitative RT-PCR studies showed that the salmon IFNs caused an increased influx of B-cells and cytotoxic T-cells at the muscle injection site, which may in part explain the adjuvant effect of the IFNs.

1. Introduction

The large increase in fish farming in the world requires adequate control of virus diseases, which is a major problem in the industry [1]. The orthomyxovirus infectious salmon anemia virus (ISAV) is an example of a virus that causes serious disease of farmed Atlantic salmon in Europe, Chile and North America. In 2007, ISAV caused an outbreak in Chile that had devastating effects on the country's salmon farming industry [1]. While most bacterial diseases of farmed salmon have been defeated by highly protective vaccines, virus vaccines based on formalin inactivated viruses or recombinant viral proteins have been unable to efficiently combat virus diseases [2]. DNA vaccines seemed to be more promising since they provide a high level of protection against the rhabdoviruses viral hemorrhagic septicaemia virus and infectious hematopoietic virus in salmonids [3]. However, DNA vaccines against ISAV and infectious pancreatic necrosis virus have shown inferior protective effects compared to rhadoviral DNA vaccines [4]. In this work we decided to study if type I IFNs (IFN-I) might be used as adjuvants in DNA vaccines against virus in fish since it is known that IFN-I enhance the adaptive immune response in mammals [5]. The adjuvant effect of IFN-I in mammals has been linked to direct stimulation of B cells T cells and dendritic cells [6,7].

Atlantic salmon possesses at least four IFN-I subtypes, named IFNa, IFNb, IFNc and IFNd, which have only 22 to 37% amino acid sequence identity and show major differences in cellular expression and antiviral activity [8–10]. IFNa1 and IFNc induce antiviral activity against IPNV and ISAV, while IFNb is less active and IFNd show no antiviral activity [9,11]. In this work we have tested the adjuvant effect of IFNa1, IFNb and IFNc in a DNA vaccine model, using ISAV hemagglutinin-esterase (HE) as antigen [12]. Atlantic salmon presmolts were injected with plasmid expressing HE alone or together with a plasmid expressing IFN, and protection and antibody levels against ISAV were the measured 7–10 weeks later. The results showed that all three IFN plasmids delivered together with HE plasmid potently enhanced protection and antibody production against ISAV. In contrast, HE plasmid alone gave low antibody titers and a minor protection against ISAV. RT-qPCR of immune genes at

Abbreviations: IFN, interferon; ISAV, infectious salmon anemia virus; IPNV, infectious pancreatic necrosis virus; HE, hemagglutinin-esterase; i.m., intramuscular; i.p., intraperitoneally; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription quantitative PCR.

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the muscle injection site suggested that the IFNs attracted B cells and cytotoxic T cells.

2. Materials and methods

2.1. Fish

Atlantic salmon (*Salmo salar* L.) presmolts (30–45 g) were obtained, labeled and kept in 300 l tanks at 10 °C as described [13]. 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. Virus

The ISAV Glesvaer/2/90 isolate was obtained and grown as described [11]. Virus titers were calculated by the $TCID_{50}$ method [14].

2.3. Plasmids used for intramuscular injection

Plasmids encoding Atlantic salmon IFNa1, IFNb and IFNc were available from a previous study [9] and were sub-cloned into the pcDNA3.3-TOPO vector (Invitrogen) downstream of the CMV promoter. In this work we named these plasmids pIFNa, pIFNb and pIFNc. A religated pcDNA3.3 plasmid without insert was used as negative control. The ISAV hemagglutinin-esterase (HE) expression vector was obtained from professor Espen Rimstad, Norwegian School of Veterinary Science, Oslo, and consists of ISAV HE fused to the N-terminus of EGFP in the eukaryotic expression plasmid pEGFP-N1 [12]. The pEGFP-N1 plasmid was used as negative control. In the present work we named these plasmids pHE and pEGFP, respectively. Plasmids were transformed and grown in One Shot TOP10 *Escherichia coli* (Invitrogen) and purified by EndoFree plasmid purification kit (Qiagen).

2.4. DNA vaccination and challenge

Presmolt salmon were injected i.m. approximately 1 cm below the dorsal fin with one or two plasmids (15 μg each) in 50 μl sterile PBS at pH 7.4 or with PBS only in three different tanks as described in Fig. 1A (Tank 1), Fig. 1B (Tank 2) and Fig. 1C (Tank 3), respectively. In Fig. 1A and B tanks, the fish were kept for 7 weeks and then infected by i.p. injection with 10^4 ISAV. In Fig. 1C tank, the fish were challenged 8 weeks post vaccination by addition of 20% "shedder" presmolts, which had been injected i.p. with 10^5 ISAV. Mortality was recorded daily until day 32 in Tank 1 and Tank 2, and until day 62 in Tank 3.

2.5. Quantitation of virus in head kidney of survival fish from the cohabitation challenge

ISAV was quantitated by RT-qPCR of segment 8 in head kidney from survival fish in the cohabitation challenge trial 62 days after addition of shedder fish (experiment 1C). Head kidneys were kept in RNAlater (Ambion) and total RNA was isolated by Qiagen RNeasy mini kit (Qiagen). One μg RNA was used for cDNA synthesis by QuantiTect Reverse Transcription Kit (Qiagen). qPCR was carried out using ISAV segment 8 primers as described [15]. Elongation Factor 1αB (EF1αB) was used as a reference gene [16]. ISAV segment 8 cloned into the pCR-Blunt II TOPO plasmid (Invitrogen) was used for calculating ISAV segment copy numbers per 0.1 μg RNA in the head kidney samples by preparing a standard curve of plasmid copy numbers versus CT-values as described in the manual from the manufacturer of the 7500 FAST Real-Time PCR system (Applied Biosystems), which was used for qPCR. Based on this, virus copy numbers in fish was plotted as a column using box and whiskers, and significant differences were analyzed by Kruskal–Wallis test and post hoc Dunn's multiple comparisons test method (GraphPad Prism vision 6.01 for Windows, San Diego).

2.6. Measurement of antibody response by ELISA

ISAV-specific IgM antibodies in salmon sera were measured by ELISA using ISAV as antigen. ISAV4 was propagated in ASK cells and purified by ultracentrifugation as described [17] using a 15 to 65% (wt/vol) sucrose gradient. The virus fraction was harvested and the total protein concentration was measured with BCA protein assay kit (Pierce, Thermo Science).

Microtiter plates (Immuno-Plate Maxisorp, Nunc) were coated overnight at 4 °C with ISAV corresponding to 200 ng protein in 100 μl 0.1 M sodium carbonate buffer (pH 9.6) in each well. The plates were then washed and blocked with 5% dry skim milk in TBST buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6). Fish sera were two-fold diluted in TBST buffer from 1:50 to 1:12,800 fold dilution and 100 μl of each dilution was added to each antigen-coated well and incubated over night at 4 °C. Monoclonal mouse anti-salmon Ig (H) (Clone IPA3D1, mouse IgG1, Cedarlane labs) (1:300) and subsequently peroxidase conjugated polyclonal goat anti-mouse Ig (Invitrogen) (1:2000) were added in TBST buffer and incubated at room temperature for 2 h. One hundred micro liter TMB substrate (Thermo Scientific Pierce) was added and reacted for 10 min when the reactions were stopped with 100 μl 2 M H_2SO_4. Optical density was measured at 450 nm. Sera from ISAV-infected fish were used as the positive control. All sera were tested in duplicates and antibody titer was determined as the highest dilution whose OD_{450} was above the mean OD_{450} plus 2 standard deviations of the same serum dilution from fish injected with control plasmids or from fish injected with HEK293 cell supernatant for oil-formulated vaccine experiment. The antibody titer of each fish was plotted as a column using scatter graph (GraphPad Prism vision 6.01 for Windows, San Diego). If the titer was lower than its starting dilution of 1:50, the titer was ascribed a value of 25 when calculating the mean titer. The mean of each group was printed above its column. Significant differences of the mean ranks between different columns were analysis by Kruskal–Wallis test and post hoc Dunn's multiple comparisons test method (GraphPad Prism vision 6.01 for Windows, San Diego).

2.7. Virus neutralizing activity

ISAV neutralizing activity in sera was tested as described by Lauscher [16].

2.8. Vaccination of fish with an oil-formulated vaccine containing inactivated ISAV with or without recombinant IFNc

Recombinant IFNc (rIFNc) was prepared in HEK293 as described [9]. ISAV was grown in ASK cells and after freezing and thawing, virus in the cell lysates were inactivated with 0.1% formalin at room temperature for 3 days. After inactivation, formalin was removed by dialysis against PBS. The antigenic content of the inactivated virus was measured by hemagglutination assay [18]. Each vaccine dose consisted of 0.1 ml containing 8 hemagglutination units of inactivated ISAV (WVV) alone or combined with 0.1 ml HEK293 medium in oil adjuvant or WVV combined with 3.6×10^4 unit rIFNc in oil adjuvant. Control vaccines contained HEK293 medium or rIFNc in oil adjuvant. The vaccines were formulated as a water-in-oil emulsion using Montanide™ ISA 763A VG (Seppic) with an adjuvant/antigen ratio of 70/30 (weight/weight). Groups of Atlantic salmon presmolts (65 fish per group,

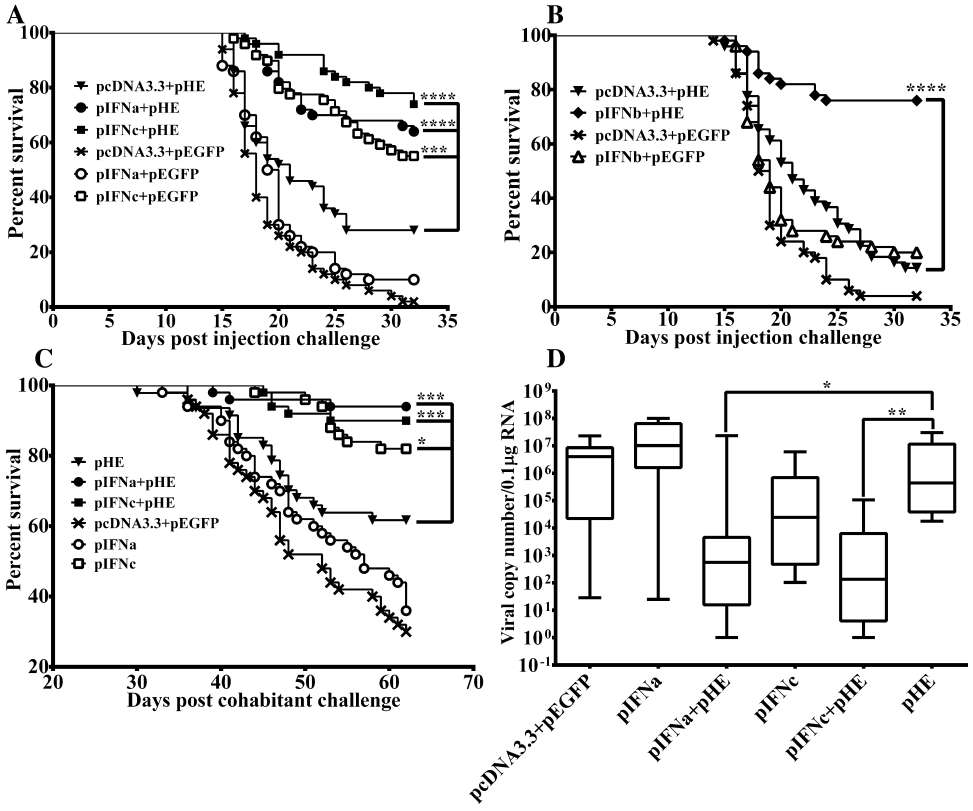

Fig. 1. Survival plots and virus quantitation for salmon vaccinated by intramuscular injection of ISAV hemagglutinin esterase plasmid (pHE) in combination with IFN plasmids after challenge with ISAV. (A) Fish challenged by i.p. injection of ISAV at 7 weeks after vaccination with pHE in combination with pIFNa1, pIFNc or pcDNA 3.3 or with pEGFP in combination with IFN plasmids or pcDNA3.3 ($n = 50$). (B) Fish challenged by i.p. injection of ISAV at 7 weeks after vaccination with pHE in combination with pIFNb or pcDNA 3.3 or with pEGFP in combination with pIFNb or pcDNA3.3 ($n = 50$). (C) Fish challenged by addition of ISAV infected cohabitant fish 8 weeks after vaccination with pHE in combination with pIFNa1, pIFNc or pcDNA 3.3 plasmids or with pEGFP in combination with IFN plasmids or pcDNA3.3 ($n = 50$). pcDNA3.3 is the plasmid control for IFN plasmids while pEGFP is the plasmid control for the HE plasmid. (D) Quantitation of ISAV in head kidney of surviving fish from the cohabitation challenge in (C) At day 62 after addition of infected cohabitants in the vaccine experiment described in (C) head kidneys were sampled from 8 survival fish in each of the vaccine groups. ISAV levels in the organs were compared between vaccine groups by RT-qPCR of ISAV segment 8, which was converted to virus particles as described in Materials and methods. Statistical significant differences between pIFNs + HE and pcDNA3.3 + HE groups are indicated with asterisks, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$.

average weight 30 g) were anesthetized with 0.005% benzocaine and tagged by alcian blue tattooing. Each fish was injected i.p. with a vaccine dose of 0.1 ml. All groups were kept in one 900 l tank in fresh water of 10 °C. Group 1 fish were injected with WVV only, Group 2 fish were injected with HEK293 cell supernatant alone, Group 3 fish were injected with WVV together with HEK293 supernatant, Group 4 fish were injected with rIFNc alone and Group 5 fish were injected with WVV together with rIFNc. Eight weeks post vaccination, sera were sampled from 15 fish before cohabitant infection by addition of 20% shedder fish, which were injected i.p. with 0.1 ml ISAV (10^5 $TCID_{50}$) and placed into the same tank. Mortality was recorded daily. The experiment was ended at day 62 p.i. when the mortality of the control group had reached approximately 70%.

2.9. Reverse transcription quantitative PCR (RT-qPCR)

Muscle samples from plasmid injection sites were collected in RNAlater (Ambion) and RNA isolation, cDNA synthesis and real time PCR were performed as described previously [13]. The relative expression of each gene was calculated by the $\Delta\Delta$Ct method [19] using EF1αB as a reference gene [16]. Data were calculated from triplicates of five samples in each group, and expressed as mean ± standard errors. The primers used in RT-qPCR are listed in Table 1. Gene transcripts were compared using an unpaired Student's *t*-test and considered as statistically significant at $p \leq 0.05$.

3. Results

3.1. IFNs increase the protective effect of HE against challenge with ISAV

To test if the IFNa1, IFNb or IFNc might increase the protective effect of HE against ISAV infection of salmon, fish were injected with various combinations of IFN plasmids and HE plasmids or the respective control plasmids, kept for 7 or 8 weeks and then challenged by ISAV (Fig. 1). In the 7 week vaccination experiment, the effects of pIFNa1 and pIFNc were studied in one tank (Fig. 1A) and the effect of the pIFNb in another tank (Fig. 1B). 32 days after injection of ISAV, fish injected with control plasmids showed high mortality in both tanks (<5% survival). In tank 1, fish injected with pHE and pIFNa1 showed significantly higher protection (64% survival) than fish injected with pHE and pcDNA3.3 (28% survival) or pIFNa1 and pEGFP (10% survival). As expected [13], pIFNc also gave significant protection (55% survival), but fish injected with pHE together with pIFNc obtained even higher protection (74% survival). In tank 2, survival of fish injected with pHE together with pIFNb was much higher (76%) than of fish injected with pIFNb and pEGFP (20%) or with pHE and pcDNA3.3 (14%).

In the 8 week vaccination experiment, the adjuvant effects of pIFNa1 and pIFNc were compared by cohabitation challenge with ISAV injected fish, which is similar to natural infection (Fig. 1C). The mortality of fish injected with pIFNa1 or control plasmids again developed similarly (36 and 30% survival) while pHE gave some

Table 1
Primers used for qPCR of immune genes.

Gene name	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
IgM	Y12457.1	TGAGGAGAACTGTGGGCTACACT	TGTTAATGACCACTGAATGTGCAT
IgT	ACX50290	CAACACTGACTGGAACAACAAGGT	CGTCAGCGGTTCTGTTTTGGA
CD3 epsilon	NM_001123622	CTCAGGGCTCGGAAGAAGTCT	GGCCACGGCCTGCTGA
CD4	EU409794.1	GAGTACACCTGCGCTGTGGA	GGTTGACCTCCTGACCTACAA
CD8a	NM_001123583.1	CGTCTACAGCTGTGCATCAATCAA	GGCTGTGGTCATTGGTGTAGTC
CD83	DQ339141.1	GCACCTGTAGGAGAGCAGAACC	TCCCTTTCTTCTGATTGGTCTGT
CD86	DW580717.1	TAGACCACACACAGGGAACAATG	ATTGAGATGTATGTTCTTGTCGTCG
MHC II	ABX44766.1	ATGGTGGAGCACATCAGCC	CTCAGCCTCAGGCAGGGAC
MHC I	JN561338.1	GAAGAGCACTCTGATGAGGACAG	CACCATGACTCCACTGGGGTAG
Perforin 1-1	ACI33854.1	CGTTGTCACCATGGAACGTAA	GCCTCTGAGCCTGTCTATCCA
Perforin 1-2	ACI33201.1	CTCCATCGCTCCAGTGAGTCT	TGGTTCCAGCGAGCATAAACT
Granzyme A	NM001141037.1	GCTAAGGGGAGTCGTGTCCTT	CAATGACTTCCGCATGGTTTT
IFN gamma	AY795563.1	AAGGGCTGTGATGTGTTTCTG	TGTACTGAGCGGCATTACTCC
EF1a	BG933853	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG

protection (60% survival). In contrast, pIFNa1 plus pHE, pIFNc plus HE or IFNc alone gave much higher protection (94%, 90% and 82% survival), which were significantly higher than in the pHE group. Virus copy numbers in head kidney of survival fish was estimated by RT-qPCR (Fig. 1D). Fish injected with control plasmids or pIFNa alone contained the highest virus numbers, which was not significantly different from fish injected with pHE alone. As expected, fish injected pIFNa + pHE or pIFNc + pHE contained significantly lower virus numbers. Taken together, these results demonstrate strong adjuvant effects of IFNa and IFNb plasmids when administrated together with the HE plasmid. The adjuvant effect of pIFNc was less clear from these challenge experiments because pIFNc alone provides such a high level of protection against ISAV infection [13].

3.2. Type I IFNs enhance the antibody response against ISAV

To test whether IFNs stimulate the antibody response of Atlantic salmon against ISAV HE antigen, the fish were injected i.m. with pHE together with either pIFNa, pIFNb, or pIFNc or with pcDNA3.3 without insert. Controls were IFN plasmids injected together with pEGFP. Ten weeks later, sera were harvested and IgM antibody responses were measured by ELISA using whole ISAV as coating antigen (Fig. 2). The results showed that antibody titers of sera from fish injected with pEGFP together with IFN-plasmids or the pcDNA3.3 control plasmid were similar and ranged from 72 to 462. The mean antibody titer of sera from fish injected with the pHE together with pcDNA3.3 was also only 412. In contrast, the mean antibody titers of fish injected with pIFNa + pHE, pIFNb + pHE or pIFNc + pHE plasmids were 9603, 8803 and 9600, respectively, which were significantly higher than antibody titers from fish injected with pHE together with pcDNA3.3 ($p < 0.05$). This showed that all three IFNs enhanced the adaptive humoral immune response against ISAV HE to a similar extent and confirmed the adjuvant activity of type I IFN in salmon. ISAV neutralizing activities in the sera were measured, but showed low titers. There were no significant differences between fish vaccinated with pcDNA3.3 + pEGFP and fish vaccinated with pHE with or without an IFN plasmid (Supplemental Table 1).

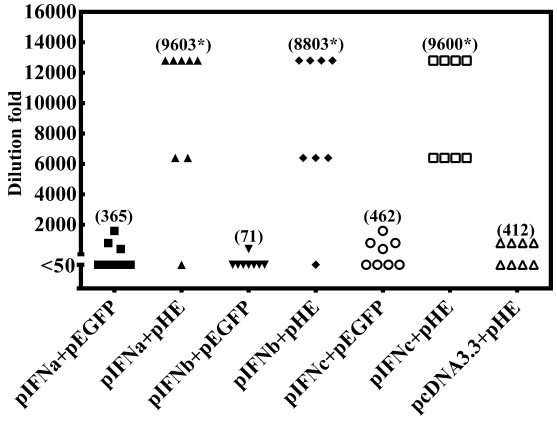

Fig. 2. Anti-ISAV antibody titers in serum of fish 10 weeks after i.m. injection of different combinations of HE and IFN plasmids. Fish immunized by injection with pcDNA3.3, pIFNa1, pIFNb or pIFNc together with pHE or by injection with pIFNa1, pIFNb or pIFNc together with pEGFP (control for HE) or by injection with pcDNA3.3 and pEGFP. IgM antibody responses were measured by ELISA using purified ISAV as coating antigen. Antibody titer was determined as the highest dilution whose OD_{450} was above the mean OD_{450} plus 2 standard deviations of the same serum dilution from fish injected with pcDNA3.3 and pEGFP ($n = 8$). Numbers above columns show mean antibody titers. Statistical significant difference between pIFNs + HE and pcDNA3.3 + HE ($p < 0.05$) is indicated with an asterisk.

3.3. Adjuvant activity of recombinant IFN with inactivated whole virus as vaccine

We also tested if recombinant IFNc (rIFNc), might increase the protective effect of inactivated ISAV as vaccine (WVV) or increase the humoral immune response against WVV. IFNc was chosen because it seemed to be more stable than IFNa *in vivo* [13] and was produced in HEK293 cells. Groups of salmon presmolts were vaccinated by i.p. injection of WVV alone, HEK293 medium alone, WVV and HEK293 medium, rIFNc alone or WVV and rIFNc, all mixed in a water in oil emulsion. Eight weeks after vaccination, sera were harvested and the fish were challenged with ISAV in a cohabitation infection model. The challenge experiment (Fig. 3A) showed that the survival of fish vaccinated with WVV plus recombinant IFNc (76%) was higher than fish vaccinated with WVV alone (66%) or WVV plus HEK293 medium (64%). Fish injected with HEK293 medium alone showed 30% survival. Antibody titers were measured by ELISA using ISAV as coating antigen (Fig. 3B). Sera from fish vaccinated with WVV alone or WVV plus HEK293 medium showed a significant increase in antibody titer when compared to fish injected with HEK293 medium only ($p < 0.05$). Sera from fish vaccinated with WVV plus rIFNc showed 3-fold higher titers ($p < 0.01$) than the groups vaccinated with WVV without rIFNc. Taken together, these results further confirmed the adjuvant activity of type I IFN in salmon.

3.4. Injection of IFN plasmids increase influx of B cells and CD8 positive cells

The outcome of DNA vaccination is likely to depend on the type of immune cells that are attracted to the muscle injection site. We thus measured the change in gene transcripts of various marker genes for B-cells (IgM, IgT), T-cells (CD3, CD4, CD8) and dendritic cells (CD83, CD86, MHCII) in the muscle at the injection site of

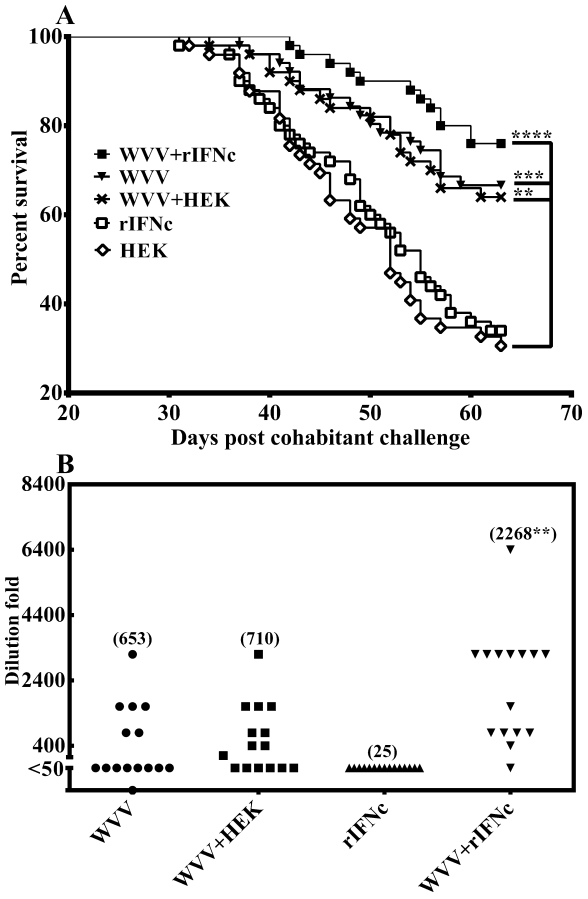

Fig. 3. Survival plots after challenge with ISAV (A) and anti-ISAV antibody responses (B) of fish 8 weeks after i.p. injection with vaccines containing inactivated ISAV (WVV) alone, WVV and medium from HEK293 cells (HEK), rIFNc alone or WVV and recombinant IFNc protein (rIFNc). A. Fish challenged by addition of ISAV infected cohabitant fish 8 weeks after vaccination ($n = 50$). Statistical significant differences were observed between the survival of the HEK group and the WVV + rIFNc group ($p < 0.0001^{****}$), between the HEK group and WVV group ($p < 0.001^{***}$) and between the HEK group and the WVV + HEK group ($p < 0.01^{**}$), but not between the WVV + rIFNc group and the WVV or WVV + HEK groups. B. IgM antibody responses were tested by ELISA using purified ISAV as coating antigen ($n = 15$). Antibody titer was determined as the highest dilution whose OD_{450} was above the mean OD_{450} plus 2 standard deviations of the same serum dilution from fish injected with HEK293 cell supernatant. The figure shows antibody titers of individuals. Numbers above columns show mean antibody titers. Statistical significant difference was observed between the WVV + rIFNc group and the WVV or WVV + HEK groups ($p < 0.01^{**}$).

IFNa, IFNb and IFNc plasmids compared to control plasmid and PBS (Fig. 4). The data showed significant increases in IgM, IgT and CD8a for all three IFN plasmids compared to control plasmid ($p < 0.05$), which suggests that IFN expression caused an increased influx of B-cells and cytotoxic T-cells. Increased transcripts were observed also for the pan T-cell antigen CD3 for all three pIFNs, while CD4 showed a minor increase for IFNb and IFNc, but not for pIFNa. Increased expression of perforin 1–2 and Granzyme A by the all three IFN plasmids support that the CD8 positive cells are cytotoxic T-cells. IFNg transcripts were increased only in muscle injected with pIFNa, however. The IFN plasmids did not seem to influence the influx of dendritic cells since there were no or minor increases in transcripts of CD83, CD86 and MHCII. MHC I was increased for all three IFN plasmids. In a separate experiment we observed that injection of HE plasmid did not cause increase in transcription of any of the genes IgM, CD8a, CD3, CD4, CD83 or CD86, MHCI or MHCII compared to injection of the control plasmid EGFP N1 (Supplemental Fig. 1).

4. Discussion

We here provide evidence that fish IFN-I have an important role in kick-starting the adaptive immune responses against virus. Strong adjuvant activity of IFNa, IFNb and IFNc plasmids was observed when injected i.m. together with a plasmid expressing ISAV HE as antigen, both with respect to protection against ISAV infection and IgM antibody response against ISAV. I.m. injection of HE plasmid alone gave some increase in protection as described in a previous work [12], but far less than in combination with IFN plasmids. Adjuvant effect was also observed for recombinant IFNc, which increased the IgM antibody response to inactivated ISAV upon i.p. delivery. While the adjuvant activity of IFN-I in mammals has been known previously, the present work shows that the link between type I IFNs and the adaptive immune system was established in fish several hundred million years ago. This work supports that IFN-I may play a role in the protective effect of DNA vaccines based on VHSV and IHNV G proteins since the latter vaccines are known to induce IFN-I and IFN-induced genes in the muscle of the fish and the VHSV G-protein has been shown to induce IFN-I in cell culture [20,21].

The protection obtained with IFNa + HE plasmids and IFNb + HE plasmids must be due to adaptive immune responses since neither IFNa alone nor IFNb plasmid alone provide any significant protection against ISAV mediated mortality [13]. On the other hand, the protection obtained with IFNc + HE plasmids is most probably due both to adaptive and innate immune responses because injection of IFNc plasmid alone provides a high level of protection against ISAV infection due to systemic induction of antiviral proteins [13]. The adjuvant effect of IFNc was, however, confirmed by its ability to enhance the antibody response against ISAV, both when delivered as gene and as recombinant protein.

The fact that IFNa, IFNb and IFNc all showed similar adjuvant effects was surprising since they have quite different properties in Atlantic salmon. The three IFNs are induced through different signaling pathways and show different expression in cells and tissues [9]. Recent work has shown that salmon IFNa, IFNb and IFNc utilize different receptors [22]. The immune cells that contribute to the adjuvant effect of IFN-I in Atlantic salmon must thus have receptors for all three IFN subtypes since they all show similar adjuvant properties. Interestingly, the IFNa1 plasmid has been shown to induces antiviral genes only at the muscle injection site, while the IFNb and IFNc plasmids induce antiviral genes systemically in the fish [13]. This means that the adjuvant effect of the IFNa plasmid is caused by stimulation of immune cells at the muscle injection site since IFNa does not induce antiviral genes systemically in salmon. Accordingly, the adjuvant activity of IFNb and IFNc is also likely to occur at the muscle injection site. Mammalian studies suggest that adjuvant activity of IFN-I is pleiotropic and due to direct stimulatory effects on T cells, B cells as well as dendritic cells, which are the main antigen presenting cells [7,23–25]. IFN-I have been shown to be important for clonal expansion of CD4 and CD8 T-cells and for initiation of cross-priming of CD8 T cells [26–28]. In addition, IFN-I have been shown to promote survival of B-cells by inhibition of apoptosis [29]. The effect of IFN-I on fish immune cells is unknown, but the present RT-qPCR studies showed that injection of all three IFN plasmids caused an increase in transcripts for IgM and IgT, which suggests an increased influx of B-cells; and an increase of CD8, perforin and granzyme A transcripts, which suggests an increased influx of cytotoxic T-cells. This might be explained by the fact that the IFNs induce several chemokine genes in the muscle tissue (unpublished data). Increased expression of MHC II was not observed for the three IFN plasmids, which suggest no increased attraction of professional antigen presenting cells by IFNs at the muscle injection site. Antigen presenting cells may still be involved in the adjuvant activity of the IFNs since these cells may be attracted

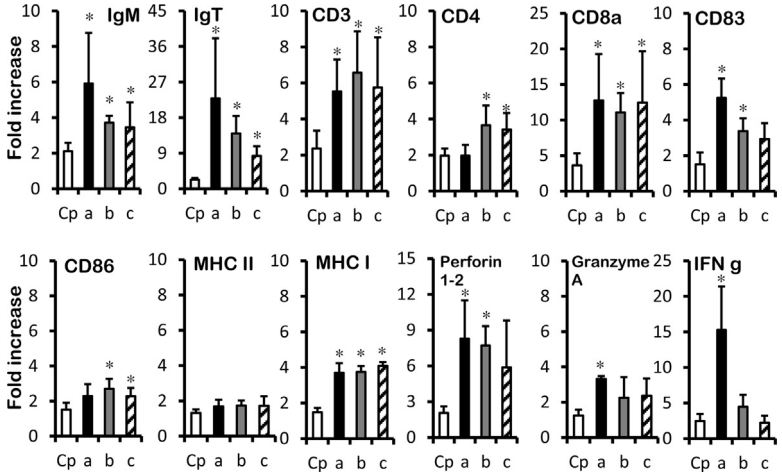

Fig. 4. Expression of immune cell marker genes in salmon presmolts after i.m. injection of IFN expression plasmids. Groups of presmolts were injected with PBS, control plasmid (Cp) or plasmids expressing IFNa1, IFNb or IFNc, respectively ($n = 5$). Gene transcription was measured by RT-qPCR 7 days after injection, and presented as fold increase (mean ± SD) compared to PBS injected fish. Statistical significant differences ($p < 0.05$) between IFN plasmid groups and control plasmid group are indicated with an asterisk.

by the wounding caused by injection. The question of whether muscle cells or professional antigen presenting cells are most important in DNA vaccination mechanism, is still debated [30,31].

The mechanism of protection obtained by injection of IFN plasmid together with the HE plasmid is uncertain since the antiserum from the vaccinated fish showed low virus neutralizing activity. Poor ISAV-neutralizing activity was also observed by antiserum from Atlantic salmon immunized with a high dose of inactivated ISAV even if the fish was highly protected against ISAV infection [16]. Non-neutralizing antibodies may contribute to protection by increased phagocytosis and destruction of virus, but this has yet to be shown for fish antibodies. The role of cytotoxic T-cells in the protective immune response has to be examined in future studies.

The present demonstration of adjuvant effects of IFN expression plasmids provides a novel method for improving DNA vaccination of fish. This is important since only DNA vaccines against fish rhabdovirus based on the G-protein have until now shown satisfactory protection against virus infection. A benefit of DNA vaccines is that they induce both humoral and cell mediated adaptive immune responses because the protein antigens are produced within the host cells [3]. Moreover, they are easy to accommodate to various virulent virus strains and can be prepared even against viruses that cannot be grown in culture. DNA vaccines are also safe to use and show less side effects than traditional fish vaccines, which have to be delivered in oil adjuvants to give a protective effect.

Conflict of interest statement

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.03.093.

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