Paper I

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Immunoprotective activity of a Salmonid Alphavirus Vaccine: Comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant

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abstract

CpG oligonucleotides and polyinosinic:polycytidylic acid (poly I:C) are toll-like receptor (TLR) agonists that mimic the immunostimulatory properties of bacterial DNA and double-stranded viral RNA respectively, and which have exhibited potential to serve as vaccine adjuvants in previous experiments. Here, a combination of CpGs and poly I:C together with water- or oil-formulated Salmonid Alphavirus (SAV) antigen preparations has been used for a vaccine in Atlantic salmon and tested for protection in SAV challenge trial. The results demonstrate that vaccination with a high dose of the SAV antigen induced protection against challenge with SAV which correlated with production of neutralizing antibodies (NAbs). As the high antigen dose alone induced full protection, no beneficial effect from the addition of CpG and poly I:C could be observed. Nevertheless, these TLR ligands significantly enhanced the levels of NAbs in serum of vaccinated fish. Interestingly, gene expression analysis demonstrated that while addition of oil suppressed the CpG/poly I:C-induced expression of IFN-J', the upregulation of IFNa1 was substantially enhanced. A low dose of the SAV antigen combined with oil did not induce any detectable levels of NAbs either with or without TLR ligands present, however the addition of CpG and poly I:C to the low SAV antigen dose formulation significantly enhanced the protection against SAV suggesting that CpG/poly I:C may have enhanced a cytotoxic response – a process which is dependent on the up-regulation of type I IFN. These results highlight the immunostimulatory properties of the tested TLR ligands and will serve as a ground for further, more detailed studies aimed to investigate their capacity to serve as adjuvants in vaccine formulations for Atlantic salmon.

1. Introduction

Pancreas disease (PD) is a serious viral disease in salmonid fish causing significant economic losses for the aquaculture industry in Europe [1]. PD is caused by Salmon Pancreas Disease Virus, now more commonly referred to as Salmonid Alphavirus (SAV) [2]. Based on sequencing and phylogenetic analysis SAV strains are grouped into 6 different subtypes: SAV1–6 [3]. SAV3 is exclusively found in Norway and affects both Atlantic salmon and rainbow trout [4,5].

Vaccines are considered to be the most effective countermeasures against diseases in aquaculture. There is a commercial vaccine available against PD based on an inactivated SAV antigen which is administrated by intraperitoneal injection (Biering, Villoing et al. [8]). Currently most fish vaccines rely on adjuvants, which improve humoral and/or cytotoxic immune responses [6,7]. In salmonid aquaculture oil-based adjuvants are the most widely used [8,9], however, due to their negative side effects, it is desirable to develop other adjuvant concepts.

It is now clear that effective adjuvants link innate and adaptive immunity by signaling through a combination of PRRs [10]. CpG ODNs, which are short synthetic DNA sequences consisting

Abbreviations: NAbs, neutralizing antibodies; Ag, antigen; ODNs, oligodeoxynucleotides; TLR, toll-like receptor; APC, antigen-presenting cells; PRRs, pattern recognition receptors; RPP, relative percent protection; HK, head kidney; RT-qPCR, reverse transcriptase quantitative real-time-PCR; GMT, geometric mean titre; SPDV, Salmon Pancreas Disease Virus; wpc, weeks post challenge; wpv, weeks post vaccination; ip, intra peritoneally.

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of unmethylated CG dinucleotides designed to specifically target TLR9 responses, are currently being developed as vaccine adjuvants [11]. In mammals CpG ODNs induce maturation of APCs [11], a process which is essential to develop an adaptive immune response. Moreover, combinations of TLR agonists can have synergistic effects when used as adjuvants, resulting in greater and more durable responses to antigens, as well as dose sparing [12,13]. We have shown that the combined treatment with CpG and poly I:C in Atlantic salmon induce synergistic up-regulation of selected immune responses [14] and also provide protection against SAV [15]. The purpose of this study was to determine whether the combination of CpG and poly I:C could modulate protective immune responses to a SAV inactivated whole virus vaccine formulated either without adjuvant or with a licensed oil adjuvant. Two different SAV Ag doses were tested which were meant to allow us to differentiate between antigen-induced responses and adjuvant-based responses.

2. Materials and methods

2.1. Reagents

Phosphorithioate-modified CpG-B ODN 2006T (T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*G*T*C*G*T*T) was purchased from Thermo Scientific (Ulm, Germany). The synthetic double stranded RNA, poly I:C, was purchased from Amersham Biosciences (Piscataway, NJ, USA). The SAV Ag formulation with or without oil adjuvant was provided by Intervet Norbio (Bergen, Norway). In this vaccine preparation the SPDV type species strain F93-125 (SAV1) was propagated in CHSE-214 anchored cells and TCID50 determined in same cells prior to virus inactivation. The water in oil emulsions were made using Montanide ISA 763 (Seppic, France).

2.2. Fish

Atlantic salmon Aquagen standard (Aquagen, Kyrksæterøra, Norway) were obtained from Tromsø Aquaculture Research Station. For the experimental challenge presmolt, approximately 70 g were used, and the fish were kept in tanks supplied with fresh water at 10 °C. Unvaccinated Atlantic salmon (about 500 g) kept in tanks supplied with filtered sea water (6–12 °C) were used for the *in vitro* study. The fish were fed commercial dry feed. Fish were euthanized using an overdose of the anaesthetic benzocaine prior to harvest of organs.

2.3. Immune stimulation and challenge

The fish were divided in eight groups and ip injected with 100 μl of one formulation per group. Formulations are described in Table1. There was no mortality observed after injection.

At 7 wpv, each treated fish were challenged with an i.p. injected dose of 5×10^3 $TCID_{50}$ SAV3.

2.4. SAV nsP1 RT-qPCR detection

To detect SAV during the viraemic phase a RT-qPCR assay was performed on viral RNA extracted from sera 1 wpc ($n = 15$) as described previously [15] using a SAV gene specific (Q-nsP1) TaqMan assay [16]. Individual *Cq*-values were transformed to relative numbers by following formula, where 20.85 is the lowest *Cq* value detected (*i.e.* has the highest number of nsP1 transcripts) and where x is any of the other *Cq* values detected:

$$Cq(x) = 2^{(20.85-x)}$$

Thereby, a sample was considered infected when it had a value between 1.0 and the cut off value of 9.7241E−06 ($x = 37.5$).

2.5. Histopathology

Samples of heart tissue were collected at 3 wpc ($n = 15$) and immediately fixed in 3.5% formaldehyde in buffered saline with pH 7.0. A scoring system was used to evaluate the severity of SAV induced heart lesions (no lesion: 0, minimal: 1, mild: 2, moderate: 3, severe: 4). Scores of 2 and more were considered to be specifically induced by a SAV infection [17]. The scoring was done as a blinded experiment.

2.6. RNA-isolation, reverse transcription and RT-qPCR of immune genes

Spleen and HK were harvested at 5 dpv ($n = 8$) and were stored in RNA later (Ambion, Applied Biosystems, USA) according to manufacturer's guidelines. RNA isolation, cDNA synthesis and RT-qPCR of different immune genes were performed as described previously [15]. The sequences of the primers and probes and the efficiencies of the assays used are presented in Table 2. The IFNa and Mx primers were designed to cover conserved regions of the IFNa1/a2 genes and Mx genes (GenBank accession numbers listed in Table 2). Relative expression was calculated using Pfaffl's mathematical model [18], where *Cq*-values were compared between saline-injected fish and vaccine-injected fish, correlated to the endogenous control EF1*aI3* and PCR-efficiency.

2.7. Virus neutralization assay

Serum samples were collected at 7 wpv and 1 wpc (8 wpv) and assayed for SAV NAbs. Hundred microliters of virus supernatant (SAV1, 6000 $TCID_{50}\,mL^{-1}$) was mixed in 96-well plates containing 100 μl of 2-fold salmon serum dilutions. Virus positive controls, using the same virus concentration, were incubated with maintenance medium, while cells incubated on maintenance medium alone were used as background controls. After 2 h the incubation mix was removed and replaced with 100 μl maintenance media. Cell cultures were incubated at 15 °C with 5% CO_2 for 8 days before SAV levels were examined by ELISA using 17H23 anti-SAV E2 [19] as primary Ab and HRP conjugated anti-mouse Ig (Bio-Rad) as secondary Ab. NAbs in serum was expressed as the highest reciprocal titers showing >50% reduction of the positive control OD value using the following equation:

$$50\%\ \text{reduction} = \frac{),\ \text{virus ctrl OD} -),\ \text{background ctrl OD}}{2}$$

NAbs titers were divided by 10 (*i.e.*, undetectable titer <20) and then transformed to binary logarithms. GMT was obtained by resulting formula:

$$\text{GMT} = \frac{(nt_{20} + nt_{40} + nt_{80} + nt_{160} + nt_{320} \ldots nt_{2560})/10}{N}^{1} \log 2$$

where n is the number of individuals/titers, N is all individuals/group, t_y is the titre and 10 is the undetectable titer <20 divided by 2.

2.8. In vitro experiment – HK leukocyte isolation and stimulation

HK leucocytes were isolated from three fish as previously described [20] and seeded at 7×10^6 cells/well in L-15 with 0.1% FBS at 14 °C. The cells were stimulated with two different concentrations of SAV High Ag, SAV High Ag CpG/poly I:C (10 and

Table 1
Trial vaccination regimen, doses and analyzes.

Treatment	Analysis (# of fish)				
	Immune gene expression 5 dpv	SAV nsP1 qPCR 1 wpc	Histopathology 3 wpc	Neutralization	
				7 wpv	1 wpc
SAV High Ag	8	15	15	14	15
SAV High Ag CpG (50 μg)/poly I:C (50 μg)	8	15	15	12	16
SAV High Ag Oil	8	15	15	15	30
SAV High Ag Oil CpG (50μg)/poly I:C (50μg)	8	15	15	15	15
SAV Low Ag Oil	8	15	15	15	20
SAV Low Ag Oil CpG (50μg)/poly I:C (50μg)	8	15	15	15	27
CpG (50μg)/poly I:C (50μg)	8	15	15	15	15
Oil CpG (50μg)/poly I:C (50μg)	8	15	15	15	15
Saline 0.9%	8	15	15	15	15

Ag, antigen; dpv, days post vaccination; wpc, weeks post challenge.

20×) and CpG/poly I:C (20×) formulations. The stimulant concentrations used were based on the amount of vaccine used in the *in vivo* trial. The overall concentration of CpGs and poly I:C in the cell culture medium was 7 and 14 μg/ml (0.93 μM and 1.86 μM CpG-B) for the 10 and the 20× stimulations, respectively. Unstimulated cells were used as control. After 24 h and 5 days the whole cell populations were harvested using RNA lysis buffer (Qiagen, Hilden, Germany). RNA isolation, cDNA synthesis and RT-qPCR were thereafter set up as described for the *in vivo* challenge.

2.9. Statistical analyses

Kruskal–Wallis Rank sum test followed by the Wilcoxon two sample *post hoc* test at a 5% level of significance were performed on the protection data (nsP1 RT-qPCR and histology). The histology test parameter used for statistical analysis was the severity of heart lesions, scored on the ordinal scale (0–4). Statistical analysis of the nsP1 RT-qPCR used the individual *Cq* values of each group as test parameters.

Kruskal–Wallis Rank sum test followed by Dunn's *post hoc* test with a 5% significance level were performed on the NAb titers. The analyses were done in GraphPad Prism 4. To evaluate significant difference regarding the immune responses performed by RT-qPCR, the relative expression software tool (REST) described by Pfaffl et al. [21] was used.

A modified expression [15] of RPP described by Beaman et al. [22] was used to evaluate the level of protection against SAV induced by the tested treatments.

3. Results

3.1. Protection

One week after challenge, during the viraemic phase, SAV-levels were detected in sera by RT-qPCR. In the saline injected group 86.7% of the fish were SAV positive, thus indicating that the challenge was successful (Fig. 1A). Interestingly, all the groups that received the High Ag dose displayed a strong protection against SAV (RPPsc. = 92–100%), including the group with Ag alone. For the two water-based High Ag formulations no virus transcript was detected, while for the two oil-adjuvanted groups one of 15 fish was SAV-positive for each of the two treatments. SAV Low Ag Oil and SAV Low Ag Oil CpG/poly I:C had reduced levels of SAV positive transcripts, where 10 fish out of 15 were SAV positive, compared to the saline controls with 13 SAV positive out of 15 fish. The RPPsc. values for the two SAV Low Ag groups were very similar (33.58 and 34.12%), however, only the SAV Low Ag Oil CpG/poly I:C was found significantly different compared to the saline group (Wilcoxon two sample *post hoc* test ($p = 0.05$)).

Table 2
Primers and probe sequences for quantitative reverse-transcriptase PCR, PCR efficiency and GenBank accession number.

Genes	Assay	Primer	Sequence (5^1–3^1)	PCR efficiency	GenBank accession #
EF1aB	Fw/Rev	Forward	TGCCCCTCCAGGATGTCTAC		BG933897
	900 μM	Reverse	CACGGCCCACAGGTACTG	2.0	
	Probe 250 μM	Probe	AAATCGGCGGTATTGG		
CXCL10	Custom	Forward	AGGAGTGTGCAGTAAATCTGTGAAC		
	TaqMan	Reverse	CTCATGGTGCTCTCTGTTCCA	2.0	EF619047
		Probe	CAATTCCACTAAGAACTTG		
IFN-*α/β3*	Custom	Forward	CCTTTCCCTGCTGGACCA		AY2169594
	TaqMan	Reverse	TGTCTGTAAAGGGATGTTGGGAAAA	1.94	AY2169595
		Probe	CTTTGTGATATCTCCTCCCATC		
IFN-γ'	Fw/Rev	Forward	AAGGGCTGTGATGTGTTTCTG		
	900 μM	Reverse	TGTACTGAGCGGCATTACTCC	2.0	AY795563
	Probe 250 μM	Probe	TTGATGGGCTGGATGACTTTAGGA		
Mx1/2	Fw/Rev	Forward	GATGCTGCACCTCAAGTCCTATTA		U66475
	900 μM	Reverse	CGGATCACCATGGGAATCTGA	2.0	U66476
	Probe 250 μM	Probe	CAGGATATCCAGTCAACGTT		
Q_nsP1	Fw/Rev	Forward	CCGGCCCTGAACCAGTT		
	450 μM	Reverse	GTAGCCAAGTGGGAGAAAGCT	–	AY604235
	Probe 250 μM	Probe	CTGGCCACCACTTCGA		

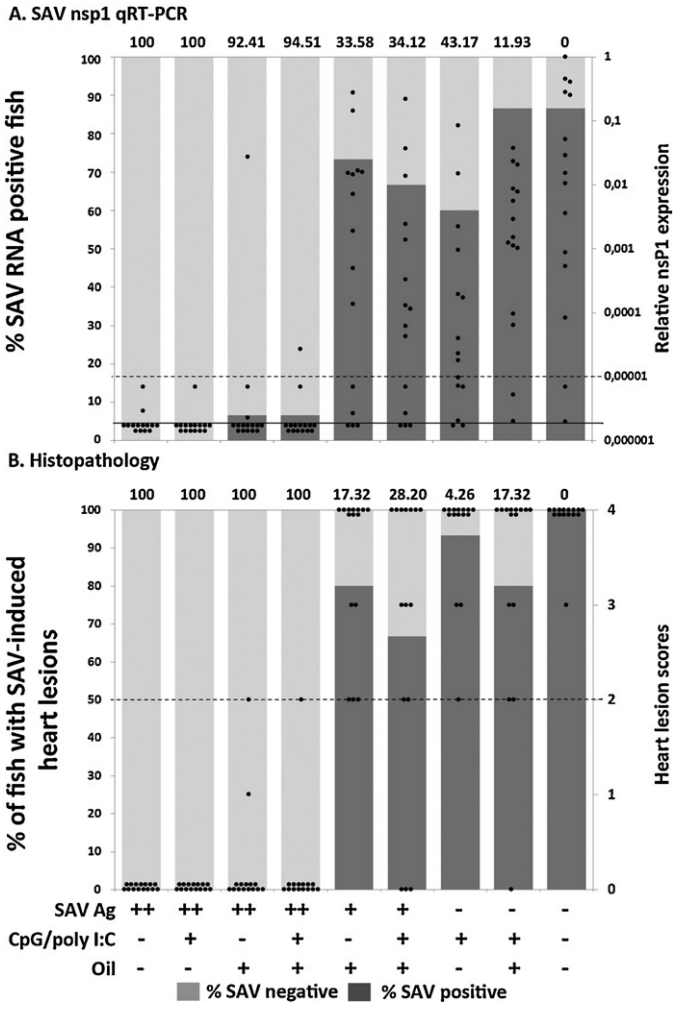

Fig. 1. Protection against SAV in vaccinated and control groups ($n = 15$). (A) Percentage of SAV RNA positive fish (left y-axis) and relative SAV nsP1 expression (right y-axis) 1 week post challenge (wpc) measured by SAV nsP1 RT-qPCR for all treatments. Individual Cq-values was transformed to relative numbers (black spots) as described in Section 2, where 1 indicates the highest presence of nsP1 transcripts. Sera below the cut off (dotted line; 9.724E−06) were considered SAV negative and the values below the solid line had undetermined Cq-values. (B) Percentage of fish with SAV3 induced heart lesions (left y-axis) and distribution of individual heart lesion scores (right y-axis) assessed by histology at 3 wpc for each treatment group. A score ≥2 was used as cut off (dotted line) for SAV specific heart lesions. Individual heart lesion scores are presented as black spots. For both graphs RPPsc. values are shown above corresponding diagram and SAV Ag ++ indicates the high dose of Ag and SAV Ag + the Low Ag dose. + or − respectively indicates presence or absence of either CpG/poly I:C or oil adjuvant.

To further determine the level of protection against PD, we assessed SAV-induced heart lesions by histology 3 wpc (10 wpv). At this time point 100% of the saline-injected fish showed SAV-specific heart lesions (Fig. 1B) where 90% of those fish displayed severe lesions, *i.e.* a score of 4. Full protection against SAV induced heart lesions (RPPsc. = 100%) was found in the four SAV High Ag groups. None of the fish injected with water-based formulations, SAV High Ag only or SAV High Ag with CpG/poly I:C, showed any lesions. For the two High Ag groups formulated with oil, 6.67–13.3% of the fish had minimal to mild lesions, *i.e.* a score of 1 or 2, and the remaining fish displayed no lesions. Among the two groups with Low Ag dose, only the SAV Low Ag Oil CpG/poly I:C treated fish presented significantly lower heart lesion scores compared to the saline-treated group (RPPsc. = 28.2%).

3.2. Vaccine-induced SAV-specific NAbs following immunization and challenge

Induction of SAV-specific NAbs in sera obtained 7 wpv and 1 wpc (8 wpv) was measured. For the pre-challenge groups only fish vaccinated with the water-based SAV High Ag doses showed detectable neutralizing titers, where 7 out of 14 tested sera in the SAV High Ag alone group and all sera in the SAV High Ag CpG/poly I:C generated NAbs (Table 3). The group with SAV High Ag alone had an average GMT of 1.52, while fish injected with SAV High Ag CpG/poly I:C had an average GMT of 7.08, which was significantly higher than all other formulations, including SAV High Ag alone (Table 3). One week after SAV challenge (8 wpv) Nabs were detected in all groups injected with a High SAV Ag dose, while only a few individuals that had received the Low SAV Ag dose produced NAbs. In the three groups without Ag no NAbs were detected. The titers had increased significantly for SAV High Ag alone, and also for SAV High Ag Oil and Oil CpG/poly I:C compared to pre-challenge titers for the same groups. The highest NAb titers was still detected in the SAV High Ag CpG/poly I:C group, however, it had no significant increase in titer upon challenge. All groups injected with SAV High Ag formulations had neutralizing activity that was significantly higher compared to the SAV Low Ag formulations and the saline treated group.

3.3. Immune genes

The results shown in Fig. 2A demonstrate that the CpG/poly I:C-only formulation strongly activated the IFN-J′ expression in HK while in spleen the response was modest and more variable (Fig. 2B). The SAV Ag did not affect the IFN-J′ expression neither in HK or spleen at this time point; however, the IFN-J′-inducing capacity of the oil formulations with CpG/poly I:C was significantly impaired in HK and to some extent reduced in spleen as well. Compared to IFN-J′, the induction of CXCL10, which is an IFN-J′-inducible gene, was substantially weaker and its magnitude was comparable between HK and spleen. Unlike IFN-J′, the expression of IFNa1 in HK was not significantly up-regulated by any of the treatments (Fig. 2C). However, the expression of Mx, a type I IFN-inducible gene, was induced in HK of fish that had received either CpG/poly I:C or Oil CpG/poly I:C, and the Mx expression was highest for both HK and spleen (Fig. 2C and D) in fish which had been injected with the Oil CpG/poly I:C formulation. Similar to the expression of IFN-J′ and CXCL10, neither IFNa1 nor Mx was significantly affected by the SAV Ag. Unlike HK, IFNa1 was up-regulated in spleen for some of the groups treated with CpG/poly I:C. However, its induction was weaker as compared to the Mx induction and there was no consistent effect of the oil on IFNa1 which is most likely due to the relatively late sampling time point, at when most of the immediately induced innate immune genes would have already been down-regulated.

3.4. In vitro experiment

The SAV Ag preparation consists of inactivated virus which may contain ligands for innate immune receptors that could possibly activate an innate immune response. Since no clear SAV-induced IFN activation was observed *in vivo*, an *in vitro* experiment was set up to check the immunostimulatory potential of the SAV formulations using a high dose of Ag alone at 24 h and 5 days. The amount of the formulations was based on the overall concentration used in the *in vivo* trial. In order to make sure that enough stimulant was applied, considerably higher concentrations were used, including 10 and 20× of the overall concentration administered in the *in vivo* trial. The CpG concentrations used (0.93 μM and 1.86 μM) are in the range known to elicit detectable

Table 3
Neutralizing serum antibodies.

Groups	Seroprevalence (%)		GMT	
	Pre challenge	Post challenge	Pre challenge	Post challenge
SAV High	50	93	1.51	2.34
SAV H/CpG/poly I:C	100	100	5.72	5.65
SAV H/Oil	7	87	–	1.42
SAV H/Oil/CpG/poly I:C	13	52	–	2.55
SAV Low/Oil	7	30	–	0.26
SAV Low/Oil/CpG/poly I:C	–	17	–	–
Saline	–	–	–	–

Sera were tested individually and their reciprocal end-point titres determined by an ELISA-method described in Section 2. Results are presented as seroprevalence (%) and the geometric mean titre (GMT) for each group using formulas described in Section 2. "–" denotes no detectable neutralizing activity (titers <20).

immune responses in *in vitro* experiments with salmon leukocytes [23]. The results shown in Fig. 3 demonstrate that while the formulations containing CpG/poly I:C elicited a strong innate immune response as indicated by the expression of IFN-*γ*, IFNa1 and Mx, the preparations with SAV Ag alone failed to induce a significant response and also, it did not affect the CpG/poly I:C-induced gene expression. After 5 days of stimulation, expression of all genes had declined and in the case of IFN-*γ* and Mx it was far below the values observed *in vivo* in HK.

4. Discussion

The major goals of the current study were (1) to characterize the potential of SAV Ag formulations adjuvanted with oil and CpG/poly I:C to induce protection against SAV infection and (2) to shed light on the mechanisms controlling the vaccine-induced adaptive immune response against the virus.

The results demonstrate that, at the high dose, the SAV Ag alone induced full protection against challenge with SAV3 at 7 wpv, while

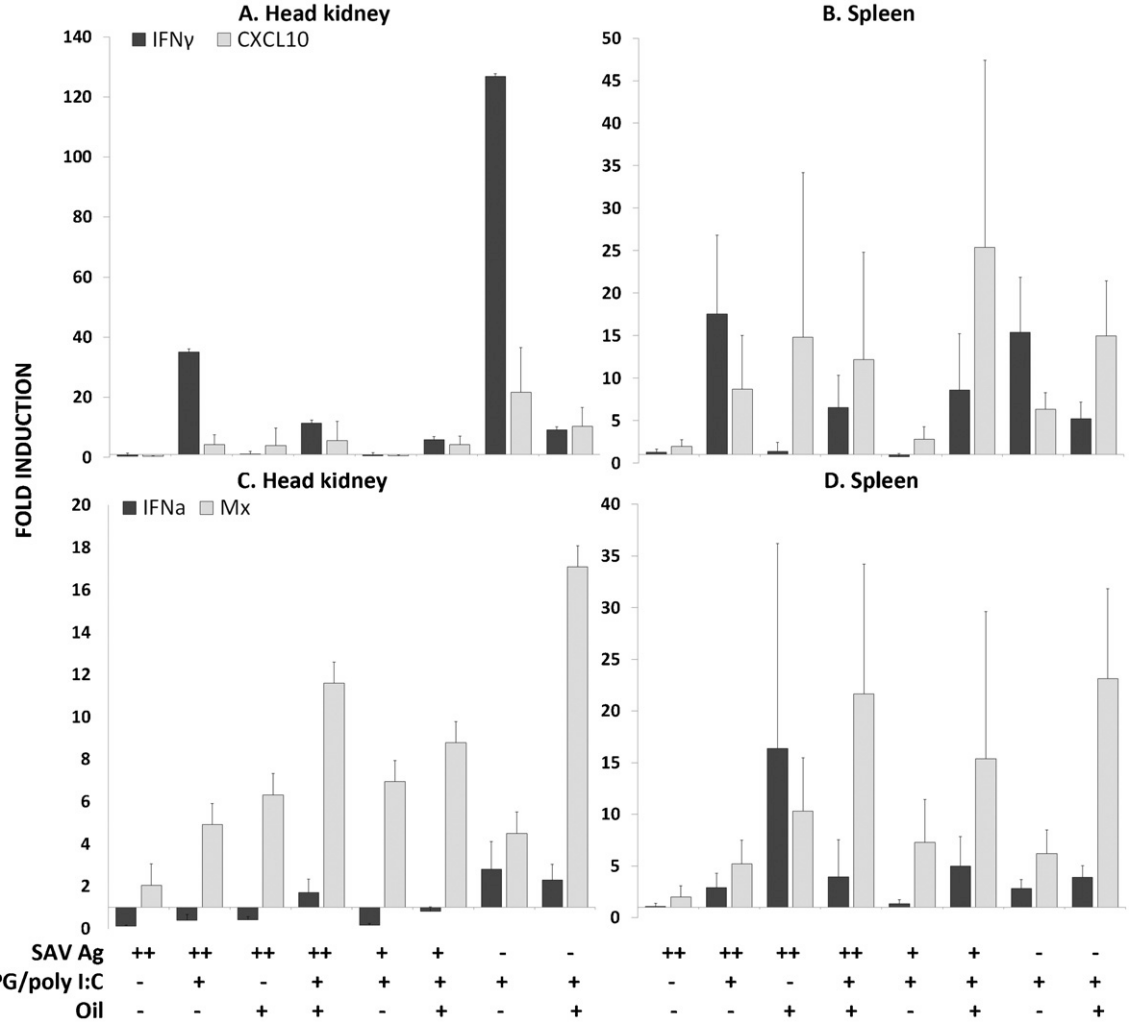

Fig. 2. Expression of IFN-*γ* and CXCL10 in head kidney (A) and spleen (B) and of IFNa1 and Mx in head kidney (C) and spleen (D) at 5 dpv measured by RT-qPCR. Results are presented as mean fold induction relative to the saline control group. The error bars represent StDev of the mean ($n = 8$). SAV Ag ++ indicates high dose of Ag and SAV Ag + Low Ag dose. + or − respectively indicates presence or absence of either CpG/poly I:C or oil.

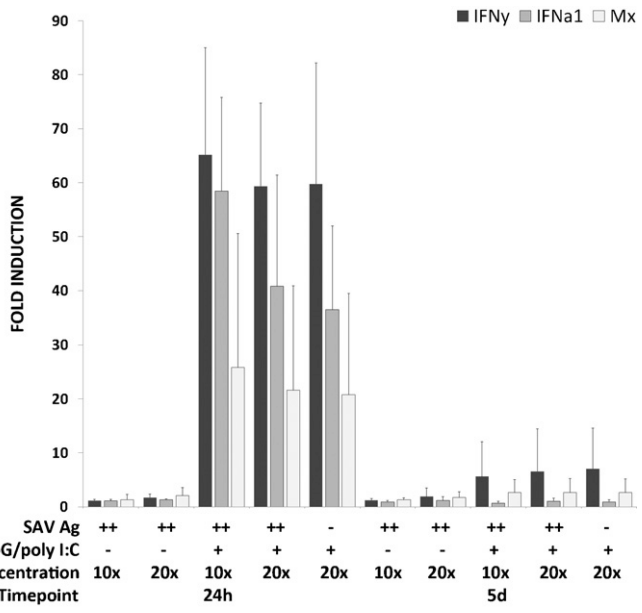

Fig. 3. Upregulation of IFN-γ, IFNa1 and Mx in head kidney leukocytes stimulated *in vitro* with the indicated formulations for 24 h and 5 days. Only the water-based formulations were tested in this experiment. The 10× and 20× concentrations indicate that 10 and 20 times higher concentrations were used as compared to the *in vivo* challenge, assuming the formulations were equally distributed throughout the fish. Results are presented as mean fold induction relative to the non-stimulated control cells. The error bars represent StDev of the mean ($n = 3$). SAV Ag ++ indicates high dose of Ag. + or − respectively indicates presence or absence of CpG/poly I:C.

the groups vaccinated with the Low Ag dose were moderately protected. Our findings demonstrate that the post-challenge viral loads in serum and the histopathological changes in heart were predictive of the Ag dose used in the vaccine formulations, with highest RPPsc. in the High Ag groups, followed by Low Ag groups, then groups with adjuvant alone and absence of protection in the unvaccinated control group. The SAV Ag itself did not induce a detectable innate immune response, as indicated by the expression of IFNa1 and IFN-γ in both the *in vivo* and in the *in vitro* experiments and the protection induced by SAV High Ag alone could be attributed to the production of NAbs as revealed by the neutralization assay. The lack of a detectable innate immune response against the SAV Ag suggests that the full protection observed in the SAV High Ag groups was most likely due to T-cell independent (TI) NAb production. SAV is an enveloped virus encoding several membrane bound glycoproteins [24], which could enable the virus to cross-link multiple antigenic receptors on specific B cells and, thereby, activate TI NAb production. Interestingly, for the pre-challenge, NAbs were substantially more abundant in the serum of the SAV High Ag CpG/poly I:C group, which could be linked to the effect of TLR ligands on the Ab secretion by B-cells, activated by multivalent TI Ags [25]. This shows that although the B-cell proliferation can be directly induced by TI-2 Ags (*e.g.* bacterial capsular polysaccharides and highly organized repetitive viral glycoprotein antigens), the provision of a second signal through innate immune receptors, such as TLRs, is necessary for an efficient Ab secretion. Reports has shown that the CpG-motif 2006 (used here) stimulate proliferation of Atlantic salmon, spleen, HK and blood leukocytes [23], indicating that it might function as a B cell mitogen in fish as well.

Oil-based adjuvants are commonly used to improve Ab production [29]. This can be explained by the protection of the oil-formulated Ags from proteolytic degradation [29], or slow Ag release over a prolonged period. Addtionally, that these adjuvants influence migration of leukocytes to the site of the Ag administration [30]. Surprisingly, including oil to the SAV High Ag vaccine formulations significantly reduced the levels of pre-challenge NAbs, and the levels of the pre-challenge NAbs could not be directly associated with the level of protection detected with RT-qPCR and histopathology. Although the amount of the pre-challenge NAbs was significantly lower in groups treated with oil-adjuvanted vaccine formulations compared to water based formulations, at 1 wpc the levels for the SAV High Ag Oil-formulations were comparable with those induced by the SAV High Ag alone formulation indicating that the response of memory B-cells was very important for the full protection induced by vaccination with the SAV High Ag dose. The positive effect of CpG/poly I:C treatment on the post-challenge NAb production, indicated by the GMT values, is also detected in the oil-adjuvanted groups, which again highlight the potential of TLR ligands to enhance Ab production.

A negative effect of the oil adjuvant on the CpG/poly I:C activity was also seen when the expression of IFN-γ was analyzed (Fig. 2) and was discussed in our previous paper devoted to the SAV protective effects on administration of CpG/poly I:C alone [15]. It is known that oil particles are actively endocytosed by phagocytic cells, such as macrophages, and DCs, and the uptake of oil has been used as an indicator for the phagocytic capacity of leukocytes [31]. This process could, potentially, restrict the availability of the dissolved ligands to non-phagocytic cells, such as NK cells which are able to respond to TLR ligands, including CpGs [32], and who are major producers of IFN-γ, along with activated T-cells [33]. A negative effect of the oil on IFN-γ production has also been observed in experiments with mice in which the IFN-γ production was suppressed in Ag-stimulated splenocytes isolated from mice immunized with a vaccine formulated with oil and CpGs [34].

Unlike IFN-γ, type I IFN can be produced by virtually all nucleated cell types [35] and in the current study, the oil formulations induced the Mx expression both in spleen and HK and the highest levels of Mx expression were detected in specimens treated with Oil CpG/poly I:C (Fig. 2). Compared to Mx, the IFNa1 up regulation was modest which could be due to the relatively late sampling point. Positive effects of oil on type I IFN production has also been observed in mammalian models [36–38]. The exact mechanism leading to the induction of type I IFN response by oil adjuvants is not clear but it may possibly be attributed to its influence on the leukocyte migration and its active uptake by phagocytes.

Elimination of virus infections by the adaptive immunity relies both on humoral and cellular immune responses. The Ag-specific cellular adaptive immune responses depend on generation of cytotoxic CD8+ T-cells and the importance of these cells for the antiviral defense is demonstrated by the association between functional exhaustion of CD8 effector cells and chronic viral infections [39]. T-cell dependent responses against protein Ag require prior activation of the innate immune response and, in particular, activation of professional APCs such as DCs [40]. CpGs and poly I:C are known to activate immature DCs and to induce their terminal differentiation/maturation [41,42]. In addition, activation and expansion of cytotoxic T cells are critically dependent on the presence of type I IFN [43]. Thus, upon infection with virus, mature DCs will present the MHC I-bound viral peptides and at the same time secrete large amounts of type I IFN leading to an efficient cellular adaptive immune response. In the current paper, both type I and II IFNs and IFN-inducible genes were induced by the CpG/poly I:C formulations and although the vaccination with SAV Low Ag Oil CpG/poly I:C did not induce any detectable levels of serum NAbs, the SAV protection in this group was significantly enhanced as compared to the saline group and was also slightly better compared to the SAV Low Ag Oil group, as detected both with SAV RT-qPCR and histopathology. Therefore, it is possible that the protective effect of the SAV Low Ag Oil CpG/poly I:C formulation could have been caused by activation of cellular immune responses. Overall, the results in the current paper highlight the potential of the tested CpG/poly I:C vaccine

formulations to enhance the production of NAbs in Atlantic salmon. Elucidating the mechanisms through which different vaccine formulations regulate expression of different types of IFN and induce cellular adaptive immune responses will provide valuable information for improving antiviral vaccines used in salmon aquaculture.

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