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DNA vaccine expressing the non-structural proteins of *Piscine orthoreovirus* delay the kinetics of PRV infection and induces moderate protection against heart -and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

Hanne M. Haatveit [a], Kjartan Hodneland [b], Stine Braaen [a], Elisabeth F. Hansen [a], Ingvild B. Nyman [a], Maria K. Dahle [c], Petter Frost [b], Espen Rimstad [a,*]

[a] Department of Food Safety and Infectious Biology, Norwegian University of Life Sciences, 0454 Oslo, Norway
[b] MSD Animal Health Innovation AS, Bergen, Norway
[c] Norwegian Veterinary Institute, 0454 Oslo, Norway

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ABSTRACT

Piscine orthoreovirus (PRV) causes heart- and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*). Erythrocytes are the main target cells for PRV. HSMI causes significant economic losses to the salmon aquaculture industry, and there is currently no vaccine available. PRV replicates and assembles within cytoplasmic structures called viral factories, mainly organized by the non-structural viral protein μNS. In two experimental vaccination trials in Atlantic salmon, using DNA vaccines expressing different combinations of PRV proteins, we found that expression of the non-structural proteins μNS combined with the cell attachment protein σ1 was associated with an increasing trend in lymphocyte marker gene expression in spleen, and induced moderate protective effect against HSMI.

1. Introduction

Heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*) is caused by *Piscine orthoreovirus* (PRV) [1]. HSMI is a prevalent viral disease in salmon aquaculture, and reported in Norway, Scotland, Chile and Canada [2–4], mainly in the seawater grow-out phase. The histopathological characteristics of HSMI are epi-, endo- and myocarditis, myocardial necrosis, myositis and necrosis of the red skeletal muscle. The accumulated mortality ranges from negligible to 20% [5]. The lesions are characterized by influx of inflammatory cells [6]. HSMI leads to significant economic losses in Atlantic salmon aquaculture. Intervention by optimized management remains a challenge, as PRV is considered ubiquitous in the marine phase of Atlantic salmon farming [7]. A virus closely related to PRV, named PRV-2, was demonstrated to be the etiological agent of erythrocytic inclusion body syndrome in Coho salmon (*Onchorhynchus kisutchi*) [8]. Infection of farmed rainbow trout by yet another PRV subtype called PRV-3 [9], is associated with both anemia and HSMI [10].

PRV belongs to family *Reoviridae*, genus *Orthoreovirus*, containing ten double-stranded RNA (dsRNA) genome segments encapsulated in a double-shelled protein capsid. The genome segments are divided into three size classes; three large (L), three medium (M) and four small (S), encoding the λ, μ and σ proteins, respectively [11,12]. Piscine erythrocytes are nucleated and shown to be major target cells for PRV [13], but PRV also infects myocytes of the heart- and skeletal muscles [14]. Influx of inflammatory cells into heart and muscle, which commences 1–2 weeks after peak viral replication, has named the disease.

Cytoplasmic, globular inclusions that resemble viral factories are formed in infected erythrocytes [13,15]. The viral factories have perinuclear localization and increase in size and decrease in numbers during the infection [16]. For *Mammalian orthoreovirus* (MRV), viral factories are formed as small punctate structures throughout the cytoplasm early after infection [17]. For both MRV and PRV, the viral μNS protein is the scaffolding protein that organizes these factories. The non-structural proteins of the reovirus is a major

* Corresponding author.
E-mail address: espen.rimstad@nmbu.no (E. Rimstad).

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targets of the cytoxic T-cell response [18] and as such could be attractive candidates for use in DNA-vaccines.

The cell attachment complex of orthoreoviruses consists of trimeric σ1 proteins associated with λ2, and distinct domains of σ1 bind to target cell receptors [19]. Following attachment to the cell, the virus internalize by receptor-mediated endocytosis followed by proteolytic cleavage of the outer capsid protein σ3 and release into the cytoplasm through association of the endosomal membrane and the outer capsid protein μ1 [20]. Further proteolytic cleavage removes the remaining outer capsid proteins, generating transcriptionally active core particles. For MRV it has been shown that monoclonal antibodies that interfere with cell attachment, endosomal release or viral uncoating, i.e. interfering with outer capsid proteins σ1, σ3 and μ1, as well as core protein λ2, can neutralize the virus [21].

PRV has resisted propagation in cell cultures, which has made production of inactivated whole-virus vaccines difficult. In experimental settings, DNA vaccination against viral diseases in salmonids such as viral haemorrhagic septicaemia (VHS), infectious salmon anemia (ISA) and pancreas disease (PD) have induced efficient protection [22–24]. A DNA vaccine against infectious hematopoietic necrosis (IHN) has been used in Canadian aquaculture since 2005 [25]. Alphavirus replicon vectors have been developed from several different mammalian alphaviruses and represent efficient tools in recombinant vaccine development [26]. A salmonid alphavirus (pSAV) replicon vector was found to induce efficient protection against ISA and PD in Atlantic salmon in experimental trials [22,23].

The present study was conducted to examine whether DNA vaccines expressing PRV non-structural proteins, alone or in combination with structural PRV proteins, would induce protection against HSMI. Both salmonid alphavirus replicon vector pSAV and conventional CMV promoter-driven PRV protein expression vectors were tested. We studied whether expression of the PRV virus factory assembly protein μNS could provide an efficient trigger of protective host immune response against HSMI.

2. Materials and methods

2.1. Plasmid constructs

The full-length open reading frames (ORFs) of PRV genes encoding λ1, λ2, λ3, μ1, μ2, μNS, σ1, σ2, σ3 and σNS, were amplified by the use of PfuUltra II Fusion HS DNA polymerase (Agilent, Santa Clara, CA, USA) from cDNA prepared in an earlier study [27]. pSAV replicon vectors [22] expressing each of these ORFs individually, and the eukaryotic expression vector pcDNA3.1 (+) (Invitrogen) expressing PRV μNS, σNS, σ1, σ3 or enhanced Green fluorescent protein (EGFP) (control), were constructed. In short, the PCR amplicons of the ORFs were either cloned into the AgeI and AscI restriction sites of the pSAV replicon (thereby removing the EGFP of the original replicon construct), or into the XbaI restriction site of pcDNA3.1. Six additional plasmids containing an epitope tag fused to the gene of interest; pSAV/σNS N-MYC, pcDNA3.1/σNS N-MYC, pSAV/σ2 N-HA, pSAV/μ2 N-HA, pSAV/λ2 N-HA and pSAV/λ3 N-HA, were also constructed for expression analysis as described earlier [27]. Primer sequences and names of plasmids are listed in Table S1. Sanger sequencing (GATC Biotech AG, Konstanz, Germany) verified all sequences.

2.2. Transfections of fish cells

CHSE-214 cells (ATCC CRL-1681, Chinook salmon embryo) were cultivated in Leibovitz 15 medium (L15, Life Technologies, Paisley, Scotland) supplemented with 10% heat inactivated fetal bovine serum (FBS, Life technologies), 2 mM L-glutamine, 0.04 mM mercaptoethanol and 0.05 mg/ml gentamycin-sulphate (Life Technologies). A total of 3 million CHSE cells were pelleted by centrifugation, suspended in 100 μL Ingenio Electroporation Solution (Mirus, Madison, WI, USA) and separately transfected with 3 μg of each the plasmids expressing λ1, λ2, λ3, μ1, μ2, μNS, σ1, σ2, σ3 or σNS using the Amaxa T-20 program. The transfected cells were diluted in 1 mL pre-equilibrated L-15 growth medium and 100 μL of the diluted cells was seeded onto gelatin-embedded cover slips (12 mm) in a 24-well plate for expression analysis by immunofluorescence microscopy. Transfections with pSAV/EGFP and pcDNA3.1/EGFP constructs were used as positive expression controls.

2.3. Immunofluorescence microscopy

Transfected CHSE-214 cells were fixed and stained using an intracellular Fixation and Permabilization Buffer (eBioscience, San Diego, CA, USA). The cells were washed in Dulbecco's PBS (DPBS) with sodium azide. Intracellular fixation buffer was added before incubation with primary antibodies, anti-λ1 (1:1000) [6], anti-μ1C (1:500) [14], anti-μNS (1:1000) [6], anti-σ1 (1:1000) [14], anti-σ3 (1:1000) [11], anti-myc (goat anti-myc antibody, Abcam; Cambridge, UK) or anti-HA (rabbit anti-HA antibody, Sigma-Aldrich; St Louis, MO, USA). Secondary antibodies were anti-rabbit immunoglobulin G (IgG) conjugated with Alexa Fluor 488 (Life Technologies, 1:400) or anti-goat IgG conjugated with Alexa Fluor 594 (Life Technologies, 1:400). Nuclear staining was performed with Hoechst trihydrochloride trihydrate stain solution (Life Technologies). The cover slips were mounted onto glass slides using Fluoroshield (Sigma-Aldrich) and images were captured on an inverted fluorescence microscope (Olympus IX81).

2.4. Vaccine preparations

The plasmid concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted in PBS to 1000 ng/μL. Samples for vaccination contained 10 μg of each plasmid construct in 50 μL (Table 1). The samples were blinded before shipment to VESO Vikan aquatic research facility (Vikan, Norway) where the challenge experiments were conducted.

Table 1
Vaccine groups.

Vaccination trial #I			
Group	Vector	Vaccine	Total amount of plasmids per fish (μg)
1	pSAV	μNS	10
2	pSAV	μNS + σNS	20
3	pSAV	μNS + μ2 + σNS + σ2 + λ1 + λ3	60
4	pSAV	μNS + μ1 + σNS + σ1 + σ3 + λ2	60
5	pSAV	μNS + μ1 + μ2 + σNS + σ1 + σ2 + σ3 + λ1 + λ2 + λ3	100
6	pcDNA3.1	μNS + σNS + σ1	30
7	pSAV	EGFP (control)	10
Vaccination trial #II			
Group	Vector	Vaccine	
1	pcDNA3.1	μNS + σNS + σ1	30
2	pcDNA3.1	μNS + σNS + σ3	30
3	pcDNA3.1	μNS + σNS	20
4	pcDNA3.1	μNS	10
5	pcDNA3.1	EGFP (control)	10
6	pcDNA3.1	PBS (control)	–

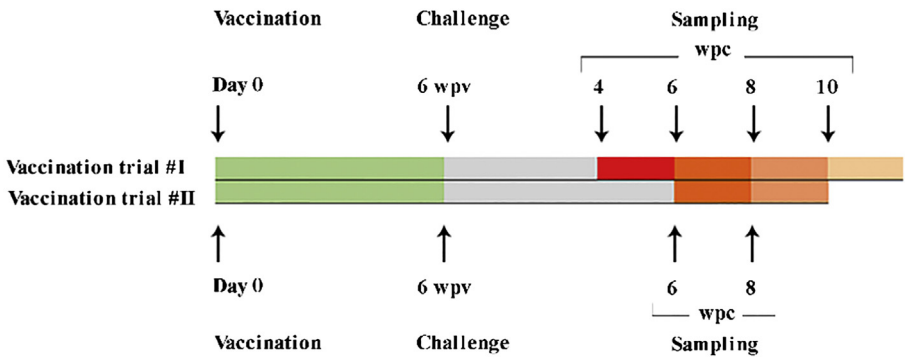
2.5. Vaccination trials

Two cohabitant challenge experiments were performed in order to evaluate the vaccine efficacy against HSMI following immunization with pSAV-based replicon vaccines and pcDNA3.1-based expression vaccines (Fig. 1). The trials were performed using unvaccinated Atlantic salmon pre-smolts with an average weight of 30–40 g, confirmed free of common salmon pathogens. The fish were kept in a freshwater flow-through system (temperature: 12 °C; oxygen: >70%; pH 6.6–6.9), acclimatized for 1 week and starved 48 h prior to vaccination. The fish were randomly selected for vaccination, anesthetized by bath immersion (2–5 min) in benzocaine chloride (0.5 g/10 L water, Apotekproduksjon AS, Oslo, Norway), labelled with passive integrated transponder (PIT) tags (two weeks prior to vaccination) and intramuscularly (i.m.) injected with the vaccines or control substances. The challenges were performed in connection with transfer to seawater six weeks post immunization. The shedders were i.p. injected with 0.1 mL of pooled heparinized blood samples from a previous PRV challenge experiment [13]. The inoculum was confirmed negative for the salmon viruses: infectious pancreatic necrosis virus, ISAV, SAV and piscine myocarditis virus by RT-qPCR. The fish were starved for 24 h prior to challenge. The experiments were approved by the Norwegian Animal Research Authority and followed the European Union Directive 2010/63/EU for animal experiments.

In vaccination trial I the fish were divided into seven groups, each containing 40 fish, and immunized by i.m. injection of 10 μg/50 μL per pSAV replicon based vaccine construct, pcDNA3.1 based vaccine construct or control pSAV/EGFP replicon (Table 1). The vaccination day was defined as Day 0. Ten untreated fish were sampled as controls prior to the experiment. Another six fish per group were sampled two and six weeks after vaccination. Six weeks after vaccination, approximately 20% PRV shedders were introduced to the challenge tank. The fish were observed daily and fed according to standard procedures. Six fish per group were sampled at 4 weeks post addition of shedder fish (wpc), 6 wpc, 8 wpc and 10 wpc, and euthanized using an overdose of anesthetics.

In vaccination trial II, the fish were divided into six groups, each containing 26 fish, and immunized by i.m. injection of 10 μg/50 μL per pcDNA3.1 construct, control construct (pcDNA3.1/EGFP) or PBS (Table 1). At 4 wpc, six fish from the PBS control group were sampled and analysed for viral RNA loads in blood to determine suitable time points for the following two samplings, set to 6 and 8 wpc. Further, 12 fish per group were sampled at these two time-points before termination of the experiment. Heparinized blood,

A



B

Fish and management	Vaccination trial #I	Vaccination trial #II
Species	Atlantic salmon (Salmo salar)	
Strain	SalmoBreed Standard	Stofnfiskur
Origin	VESO Vikan Hatchery	
Average weight	25-35 grams	
Physiological status	Presmolts	
Number of fish	290 + 20 % shedders	166 + 20 % shedders
Salinity	Fresh water during immunization/Salt water during challenge	
Stocking density	Max 50 kg/m^3	
Temperature	12°C ± 1°C	
Flow	Adjusted to 70% oxygen saturation	
Water discharge	Tube overflow system	
Cleaning	Once a day	
Photoperiod regime	L:D = 12:12 followed by 24:0	
Feeding	Automatic feeder	
Vaccination	Day 0	
PRV challenge	6 wpv	
Sampling	4, 6, 8 and 10 wpc	6 and 8 wpc
Fish sampled per time-point	6	12

Fig. 1. Vaccine trial setup. (A) The time-course and sampling points for the two vaccination trials. (B) Parameters of the two experimental trials. wpv = weeks post vaccination, wpc = weeks post challenge.

plasma and heart (stored in 4% formalin or RNAlater) were sampled from both challenge experiments.

2.6. RNA isolation and RT-qPCR

Total RNA was isolated from 20 μL heparinized blood homogenized in 650 μL QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using 5 mm steel beads, TissueLyser II (Qiagen) and RNeasy Mini spin column (Qiagen) as recommended by the manufacturer. RNA quantification was performed using a NanoDrop ND-1000 (Thermo Fisher Scientific). For the plasma samples, a 10 μL volume was diluted in PBS to 140 μL and used in the Mini spin column (Qiagen).

The Qiagen OneStep kit (Qiagen) was used for RT-qPCR with a standard input of 100 ng (5 μL of 20 ng/μL) of the isolated total RNA per reaction. From the plasma samples, 5 μL was used. The template RNA was denatured at 95 °C for 5 min prior to RT-qPCR targeting PRV gene segment S1 (S1Fwd: 5′TGCGTCCTGCGTATG GCACC′3, S1Rev: 5′GGCTGGCATGCCCGAATAGCA′3 and S1probe: 5′-FAM-ATCACAACGCCTACCT′3-MGBNFQ) using the following conditions: 400 nM primer, 300 nM probe, 400 nM dNTPs, 1.26 mM $MgCl_2$, 1:100 RNase Out (Invitrogen) and 1× ROX reference dye. The cycling conditions were 50 °C for 30 min and 94 °C for 15 min, followed by 35 cycles of 94 °C/15 sec, 54 °C/30 sec and 72 °C/15 sec in an AriaMx (Agilent, Santa Clara, CA, USA). All samples were run in duplicates, and a sample was defined as positive if both parallels produced a Ct value below 35. A quantitative PCR was also set up using target copy number in the range 10^1–10^8.

The primers used for expression analyses in spleen samples of RIG-1, Mx, PKR, ISG15, Viperin, CD8α, CD4, IFNγ, Perforin1a, Granzyme A, sIgM and mIgM are listed in Table S2 [45–50]. Elongation factor 1α (EF1α) served as an internal reference gene. The cycling conditions were 40 cycles of 95 °C/15 sec, 60 °C/30 sec and 72 °C/30 for all assays. Melting curve analyses were performed for each SYBRGreen assay.

2.7. Histopathological scoring

Sections for histopathology were processed and stained with hematoxylin and eosin. Individual fish from were examined for heart lesions in consistence with HSMI, discriminating between epicardial and myocardial changes. The grade of changes was scored from 0 to 4 (continuous) using criteria described in Table S3. The mean histopathological score ± SD at each sampling (n = 6 or n = 12) was calculated for both epicardial and myocardial changes.

2.8. Statistical analyses

The PRV RT-qPCR results and the histopathology scores were analysed statistically using the Mann Whitney compare ranks test due to the small sample sizes (n = 6/12). The Immune gene data were analysed using one-way ANOVA with Dunnetts multiple comparisons test. All statistical analysis were performed with GraphPad Prism (GraphPad Software inc., USA) and p-values of $p \leq 0.05$ were considered as significant.

3. Results

3.1. Expression in CHSE cells

All plasmid constructs expressed PRV proteins in transfected CHSE cells (Table S4). Expression of the μNS and σ1 proteins are shown in Fig. 2. The μNS protein formed small punctuate structures throughout the cytoplasm, while σ1 had an even, diffuse, distribution pattern. The pcDNA3.1 constructs yielded approximately 40% positive cells as estimated visually. This indicated better transfection efficacy of the pcDNA3.1 than the pSAV replicon constructs which gave approximately 15% positive cells. For pSAV constructs with the largest inserts only 5–10% transfection efficacy were achieved.

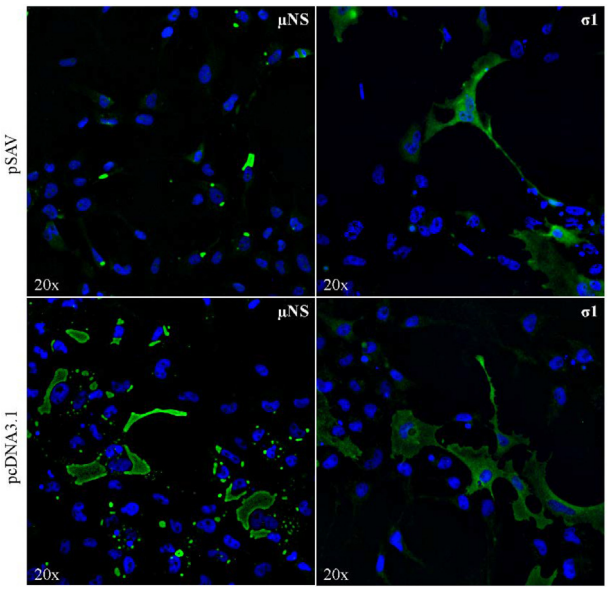


Fig. 2. Expression of μNS and σ1 in CHSE cells. CHSE cells expressing the non-structural protein μNS and the structural protein σ1 after transfection with pSAV replicon constructs (top row) or pcDNA3.1 constructs (bottom row). The cells were processed for fluorescence microscopy 96 h post transfection and the nuclei were stained with Hoechst (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Vaccination trial I

3.2.1. pcDNA3.1 expressing μNS + σNS + σ1 significantly reduced PRV loads in blood

The mean PRV Ct values in blood cells from all groups in vaccination Trial I are shown in Fig. S1. PRV was first detected in blood at 4 wpc in all groups. In the pSAV/EGFP control group, PRV RNA levels were high, peaking at 6 wpc with a mean Ct of 14.8 (±1.3) and remained high until the end of the study at 10 wpc. This verified infection kinetics in line with previous cohabitant PRV challenge experiments [28]. Similarly, the viral RNA loads in blood from all five groups vaccinated with pSAV replicon-based constructs were in the same range as the pSAV/EGFP control group. pSAV replicons encoding all ten PRV proteins (pSAV/μNS + μ1 + μ2 + σNS + σ1 + σ2 + σ3 + λ1 + λ2 + λ3; Group 5) showed delayed PRV kinetics with a peak load of viral RNA at 10 wpc (Ct of 16.4 (±2.5), Fig. 3A). At 6 wpc, Groups 2, 3 and 5 all had significantly lower viral RNA load (p-values of 0.048, 0.023 and 0.026, respectively), than the control group (Fig. S1). Group 6, vaccinated with pcDNA3.1 constructs encoding the two non-structural proteins μNS and σNS and the cellular attachment protein σ1, showed lower viral RNA loads throughout the challenge, and at 8 wpc, the mean PRV Ct (25.1 (±5.4)) was significantly higher (p = 0.002) than in the control group (16.6 (±1.5)) (Fig. 3B). The viral kinetics for this group was also delayed compared to the control group, and PRV levels did not peak in blood until the end of the challenge (10 wpc, Ct of 20.1 (±4.5)). The standard curve for the quantitative PCR is shown in Fig. S2.

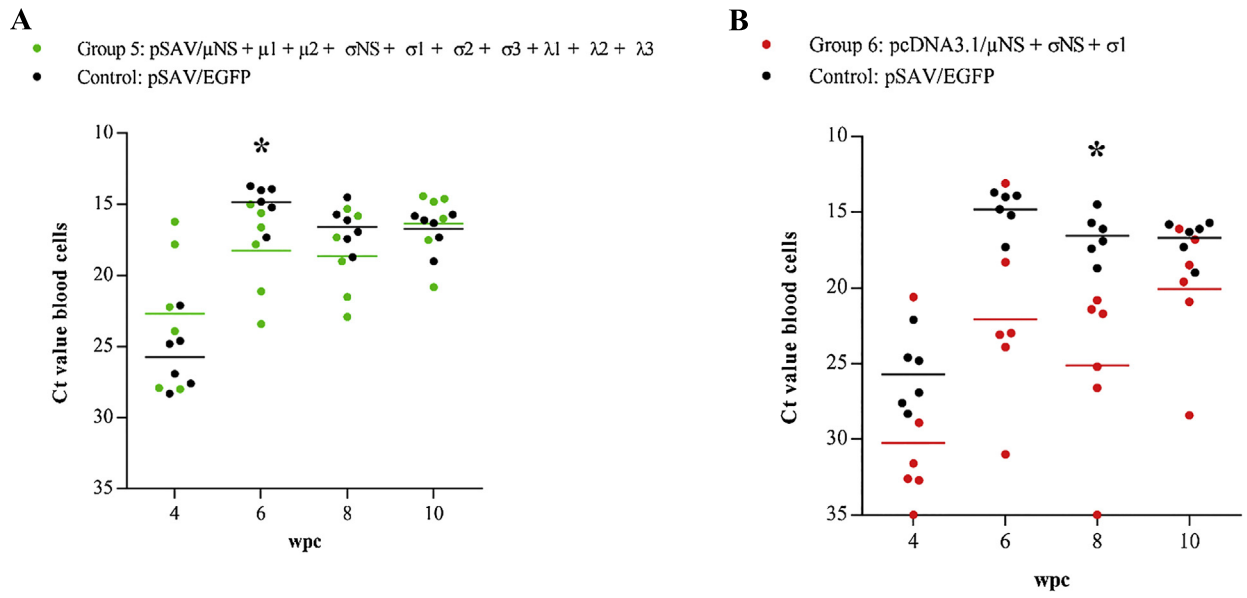


Fig. 3. Trial I: Viral (PRV) RNA level in blood cells. RT-qPCR of PRV gene segment S1 in blood cells from cohabitant fish in Trial I. (A) Group 5 (pSAV/μNS + μ1 + μ2 + σNS + σ1 + σ2 + σ3 + λ1 + λ2 + λ3) and pSAV/EGFP control. (B) Group 6 (pcDNA3.1/μNS + σNS + σ1) and pSAV/EGFP control. Cts of individual fish (dots) and mean (line). * = $p \leq 0.05$, n = 6 per group per sampling, wpc = weeks post challenge.

3.2.2. pcDNA3.1 expressing μNS + σNS + σ1 significantly reduced HSMI histopathological lesions

Fig. 4A shows the mean histopathological scores from vaccinated and control groups from Trial I. Histopathological lesions in the heart typical for HSMI were present in the pSAV/EGFP control group at 6 wpc, peaked at 8 wpc with a mean score of 2.7 and 3.3 in the epicardium and ventricle respectively (Fig. 4B). The lesions gradually resolved towards 10 wpc. The heart lesions were characterized by massive epicarditis and infiltration of lymphocytic cells in the compact and spongy myocardium layer of the ventricle. All vaccinated groups had lower heart pathology scores compared to the control group at its peaking point, 8 wpc. Group 1 (pSAV/μNS) and Group 2 (pSAV/μNS + σNS) both peaked in heart pathology 8 wpc, like the control group, while Groups 3, 4 and 5 (pSAV/μNS + various structural proteins) peaked at 6 wpc. Group 6, pcDNA3.1 expressing μNS + σNS + σ1, had reduced heart pathology in both the epicardium and the ventricle at all sampling points post challenge. At 6 wpc only one individual fish had heart lesions, and absence of heart lesions (score of 0.0) ($p = 0.002$) in both compartments was found at 8 wpc, the time when the pSAV/EGFP control group peaked (Fig. 4C). At 10 wpc, 2 out of 6 fish in Group 6 showed histopathological changes with a mean score for the group of 0.5 for both epicardium and ventricle (Fig. 4C).

3.2.3. Expression of immune genes

Immune gene expression was examined in spleen samples by RT-qPCR at Day 0 and at 6 wpv, prior to PRV exposure. Genes included in the analyses were all previously shown to be induced during PRV infection *in vivo* [40,41,46]. HSMI is characterized by influx of CD8-positive cells in the myocardium, expressing Perforin1-2 and Granzyme A [41], and production of specific anti-PRV IgM [42]. Group 4 (pSAV/μNS, μ1, σNS, σ1, σ3, λ2); Group 6 (pcDNA3.1/μNS + σNS + σ1) and the control group (pSAV/EGFP) were tested and compared to unvaccinated fish. Genes involved in innate antiviral responses, i.e. RIG-1, Mx, PKR, ISG15, Viperin and IFNγ, was induced by DNA vaccination independent of presence of PRV antigens (Table S5). For genes involved in the acquired immune response, i.e. CD8α, CD4, Perforin1-2, Granzyme A, soluble IgM and membrane IgM, there were significantly higher expression ($p < 0.05$) of CD4 after vaccination in Group 6 immunized with pcDNA3.1/μNS + σNS + σ1 only (Fig. 5). There was also a trend towards higher gene expression of CD8α, Granzyme A and soluble IgM in Group 6 compared to the other groups (Fig. 5).

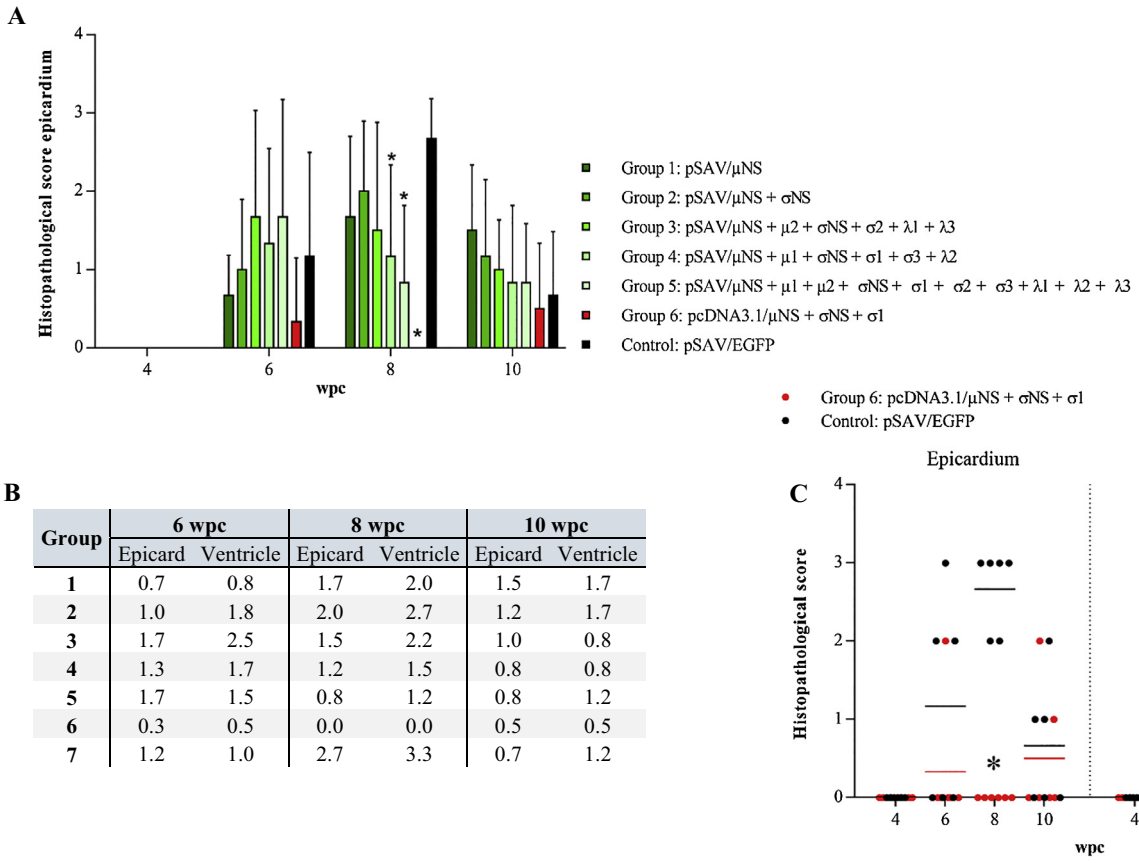
3.3. Vaccination trial II

3.3.1. pcDNA3.1 vaccine expressing μNS + σNS + σ1 reduced virus RNA level in blood

In vaccine trial II, RT-qPCR analysis revealed peak PRV loads at the two sampling points at 6 and 8 wpc for both control groups (pcDNA3.1/EGFP and PBS) with a mean Ct of 16.1 (±3.3) at 6 wpc for the pcDNA3.1/EGFP group and a mean Ct of 17.1 (±0.9) at 8 wpc for the PBS group. All vaccination groups showed reduced viral RNA loads in blood cells compared to the controls at 6 wpc (Fig. S3A). Group 1 (pcDNA3.1/μNS + σNS + σ1) showed significantly lower viral RNA load 6 wpc with a mean Ct of 24.2 (±5.3) ($p = 0.012$ and $p = 0.035$ compared to the pcDNA3.1/EGFP and the PBS groups, respectively), before peaking with a Ct of 18.7 (±1.8) at 8 wpc (Fig. 6).

3.3.2. pcDNA3.1 vaccine expressing μNS + σNS + σ1 reduced virus RNA level s in plasma

In general, the pattern of viral RNA levels in plasma from vaccination trial II was similar to that of the viral RNA levels in blood, for all vaccination groups (Fig. S3B). The pSAV/EGFP control group peaked at 8 wpc with a mean Ct of 26.0 (±1.2) and the PBS group peaked at 6 wpc with a mean Ct of 26.7 (±6.4). Group 1 (pcDNA3.1/μNS + σNS + σ1) had significantly reduced PRV RNA levels in plasma at both 6 and 8 wpc, with a mean Ct of 32.8 (±3.5) at 6 wpc ($p < 0.0001$ and $p = 0.011$), and 28.0 (±2.5) at 8 wpc ($p = 0.021$ and $p = 0.013$), compared to the pcDNA3.1/EGFP and the PBS groups, respectively (Fig. 6). At 6 wpc, Group 2 (pcDNA3.1/μNS + σNS + σ3) and Group 3 (pcDNA3.1/μNS + σNS), also showed significantly reduced viral RNA levels in plasma with Cts of 30.0 (±4.8) and 31.1 (±4.3), compared to the pcDNA3.1/EGFP ($p = 0.014$ and 0.007, respectively) and the PBS ($p = 0.023$ and 0.013, respectively) control groups (Fig. S3B).



Group	6 wpc		8 wpc		10 wpc	
	Epicard	Ventricle	Epicard	Ventricle	Epicard	Ventricle
1	0.7	0.8	1.7	2.0	1.5	1.7
2	1.0	1.8	2.0	2.7	1.2	1.7
3	1.7	2.5	1.5	2.2	1.0	0.8
4	1.3	1.7	1.2	1.5	0.8	0.8
5	1.7	1.5	0.8	1.2	0.8	1.2
6	0.3	0.5	0.0	0.0	0.5	0.5
7	1.2	1.0	2.7	3.3	0.7	1.2

Fig. 4. Trial I: Histopathological scores of epicardium and ventricle. (A) Bars illustrating mean histopathological score with SD in epicardium and ventricle. (B) Table showing mean histopathological score in epicardium and ventricle. (C) The individual histopathological scores (dots) and mean (line) in epicardium and ventricle from fish in Group 6 (pcDNA3.1/μNS + σNS + σ1) and the control group (pSAV/EGFP). * = $p \leq 0.05$, n = 6 per group per sampling, wpc = weeks post challenge.

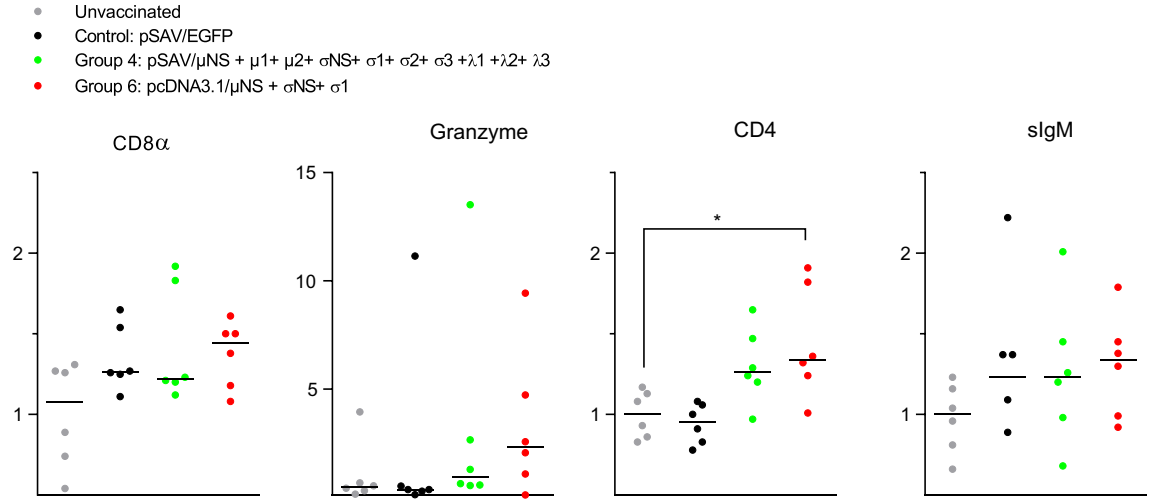


Fig. 5. Trial I: Expression of immune genes in spleen post vaccination. Genes related to the acquired immune response analysed in spleen by RT-qPCR. Samples were collected at 6 wpv, prior to PRV challenge. The results were normalized against expression of elongation factor 1α (EF1α) and compared to mean levels prior to vaccination using the ΔΔCt method. * indicates significant difference in vaccinated vs. unvaccinated groups ($p < 0.05$).

3.3.3. pcDNA3.1 vaccine expressing μNS + σNS + σ1 reduced histopathological lesions in heart

Histopathological lesions in the heart typical for HSMI were present in all fish from the control groups at 8 wpc, with a mean histopathological score of 2.5 (±0.2) and 4.0 (±0.0) in the epicardium and the ventricle, respectively, for the pSAV/EGFP group, and 2.1 (±0.4) and 4.0 (±0.0) for the PBS group. Mean histopathological scores for all groups from vaccination trial II are shown in Fig. 7. Group 1 (pcDNA3.1/μNS + σNS + σ1) showed significant reduced histopathological lesions 8 wpc with mean score of 1.0 (±0.6) ($p \leq 0.0001$) and 2.5 (±1.5) ($p = 0.0053$) in the epicardium and ventricle, respectively. Groups 2, 3 and 4 also

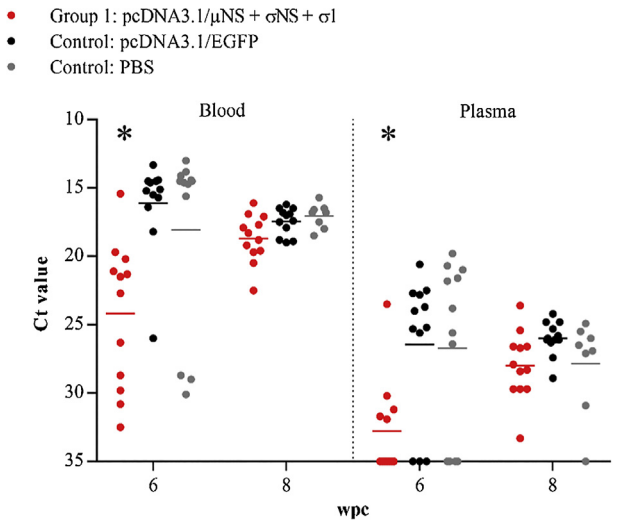


Fig. 6. Trial II: Viral (PRV) RNA level in blood cells and plasma. RT-qPCR analysis of PRV gene segment S1 in blood cells (left) and plasma (right) from cohabitant fish in Group 1 (pcDNA3.1/μNS + σNS + σ1) and the two control groups (pSAV/EGFP and PBS) in vaccination trial II. Ct values of individual fish (dots) and mean levels (line) are shown. * = $p \leq 0.05$, n = 12 per group per sampling, wpc = weeks post challenge.

showed significant reduced histopathological scores in ventricle at 8 wpc.

4. Discussion

Expression of the PRV non-structural proteins μNS, σNS, and the cell attachment protein σ1 under the control of a CMV promoter delayed the kinetics of PRV infection and induced moderate protection against HSMI in Atlantic salmon. The plasmid-based vaccine was tested in two experimental infection trials, inducing protection against HSMI in both trials. Various combinations of DNA-layered alphavirus-replicon constructs were also tested in Trial I, where PRV μNS was expressed alone or in combination with σNS and structural PRV proteins. All vaccine combinations reduced HSMI-specific heart lesions compared with the control group. However, the group vaccinated by the pcDNA3.1/μNS + σNS + σ1 combination was better protected than any of the groups vaccinated by replicon constructs. Consequently, in Trial II, various constructs using the pcDNA3.1 backbone were compared.

All pSAV replicon- and pcDNA3.1 constructs were investigated for expression of PRV proteins in fish cells before tested *in vivo* in the vaccination trial. The CHSE cell line where expression of pSAV replicon constructs has been described previously [29], was used for *in vitro* expression. Fluorescent microscopy showed that more cells were positive for the pcDNA3.1 constructs than for the pSAV replicon constructs. The size of the pSAV replicon backbone (12,073 bp) compared to the pcDNA3.1 backbone (5434 bp) would influence transfection efficiency as larger DNA constructs generally are less efficiently transfected [30]. The amount of PRV protein expressed in the transfected cells increased from 24 h until 96 h post transfection regardless of the expression vector. This is consistent with earlier studies, which have shown that expression from pSAV replicon constructs peak at day 4 post transfection [29,31]. Previous studies have shown that expression of EGFP from pSAV/EGFP transfected CHSE-214 cells is delayed and lower compared to the reporter expressed under the control of the CMV promoter [31]. The delay in protein production from pSAV constructs could be explained by the pSAV expression mechanisms, which require transcription and translation of the alphaviral replicase complex and transcription of the copy strand of the genome before transcription of the subgenomic ORF containing the PRV coding sequences.

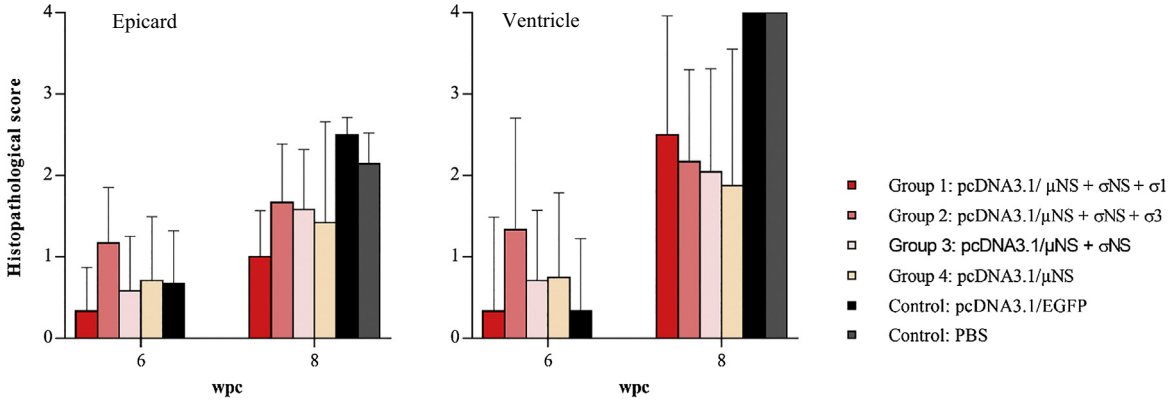
All vaccine combinations of the pSAV-replicon constructs contained the gene for the non-structural μNS protein, the organizer of viral factories [27]. The μNS forms dense, globular, cytoplasmic inclusions [16], which decrease in number and increase in size during infection [27]. The pSAV/μNS construct alone (used in Trial I, Group 1) gave typical μNS inclusions in transfected CHSE-214 cells. When used as a vaccine, this plasmid delayed the kinetics of PRV infection. Trial I Group 2, where pSAV/σNS was combined with pSAV/μNS, also delayed the kinetics of the PRV infection. The functional properties of the PRV σNS protein has not been studied, but MRV σNS associates with μNS and facilitates the assembly of virus particles [32,33]. In the Trial I Groups 3 and 4, the pSAV constructs for core proteins; λ1, λ3, μ2 and σ2, and the outer capsid proteins; λ2, μ1, σ1 and σ3, respectively, were included in addition to the pSAV/μNS and pSAV/σNS. Neither of these antigens could reduce the PRV RNA levels in blood after infection compared to the control group, but cardiac histopathological scores was reduced. In the Trial I Group 5, all the primary ORFs of the PRV genomic segments were expressed, and although the viral RNA levels were not significantly reduced compared to the control group at 8 wpc, the histopathological score was significantly lower than that of the control group at the time of maximum change, i.e. 8 wpc. In conclusion, although the various combinations of the pSAV replicon construct expressing non-structural and structural PRV proteins induced some protection against HSMI, a promising role of μNS as vaccine antigen was indicated. Reduced immunogenicity to individual components of pooled plasmid mixtures compared to single plasmid injection has been observed in DNA vaccination for both single and separate site injections, and this could influence the results for the pooled plasmid mixtures [34,35].

Trial I Group 6 was vaccinated with pcDNA3.1 vector expressing μNS, σNS and σ1 controlled by a CMV promoter. For this group the viral RNA load was significantly reduced and HSMI histopathological changes almost completely abolished. In Trial I, the combination μNS, σNS and σ1 was also part of the pSAV replicons Groups 4 and 5, which only induced some protection, again indicating that the type and number of different expression vectors may be important. Furthermore, the amount of expressed protein was higher for the pcDNA3.1 constructs than for the pSAV replicon constructs in CHSE cells, and expression levels *in vivo* may be of importance for the protection against HSMI.

Consequently, the experimental challenge Trial II was set up with pcDNA3.1 expression vectors and a limited number of plasmid variants per injection. In the Trial II Group 1, expression of μNS, σNS and σ1 were controlled by a CMV promoter, i.e. similar to Trial I Group 6. In this group the maximum viral RNA levels in plasma at 6 wpc was lower compared to the control groups, and heart pathological lesions at 8 wpc were reduced. The challenge Trials I and II were set up with similar experimental conditions, but environmental factors cannot be completely controlled and might have affected the outcome. Control fish in both experimental challenges showed similar infection kinetics following the cohabitant infection, confirming equal infection efficiency. Although more fish were sampled in the second experimental challenge, they were only sampled at two predefined time points, which might have led to information loss regarding viral kinetics.

In Trial II, only Group 1 vaccinated with pcDNA3.1/μNS + σNS + σ1 showed a delay in PRV infection kinetics (Fig. 6). Compared to the control groups, the viral RNA load in plasma was significantly reduced at 6 wpc and partly reduced in both blood and plasma at 8 wpc. Trial II Group 1 also experienced protection against HSMI at 8 WPC compared to the control groups (Fig. 7). The Trial II Group 4 was immunized with pcDNA3.1/μNS alone. This vaccine did not reduce viral RNA loads in blood cells or plasma, however,

A



B

Group	6 wpc		8 wpc	
	Epicard	Ventricle	Epicard	Ventricle
1	0.3	0.3	1.0	2.5
2	1.2	1.3	1.7	2.2
3	0.6	0.7	1.6	2.0
4	0.7	0.8	1.4	1.9
5	0.7	0.3	2.5	4.0
6	0.0	0.0	2.1	4.0

C

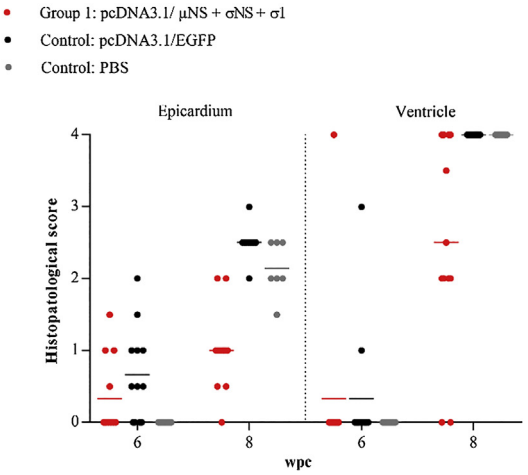


Fig. 7. Trial II: Histopathological scores in the epicardium and ventricle. (A) Bars illustrating mean histopathological score in epicardium and ventricle from in vaccination trial II. (B) Table showing mean histopathological score in epicardium and ventricle. (C) The individual histopathological scores (dots) and mean (line) in epicardium and ventricle from fish in Group 1 (pcDNA3.1/μNS + σNS + σ1) and the control groups (pcDNA3.1/EGFP and PBS).

histopathological lesions were reduced in the heart at 8 wpc, indicating that μNS alone could mount a protective response.

When μNS was expressed in combination with σNS and the outer capsid protein σ3 (Trial II Group 2) or in combination with σNS only (Trial II Group 3), no additional protective effect was obtained compared to Group 4 expressing μNS alone. This indicates that μNS and σ1 are the most promising PRV antigens for DNA vaccine development.

The vaccines expressing μNS, σNS and σ1 might have been effective compared to the other combinations due to the combination of the effect of high expression of intracellular PRV non-structural proteins and the exposed outer capsid receptor-binding σ1 protein, which is a promising target for antibodies. The mechanism of protection after successful vaccination against viral infection in fish is not fully understood, and most likely depend on both the humoral and cellular adaptive respopnses. Vaccination studies on IPN, PD and ISA claimed good correlation between titer of neutralizing antibodies and protection, indicating that the humoral immune response might be important for protection against these diseases [36–38], however, a study on ISA showed strong correlation between survival and the cell mediated immune response [39]. Previous gene expression analysis have indicated that PRV infection induces gene markers of both humoral (IgM, CD4), and cellular immunity (CD8, perforin, granzyme) [40,41]. Bead-based assay for detection of specific antibodies against PRV proteins have demonstrated a distinct increase in specific antibodies against μ1C and μNS in plasma of Atlantic salmon during the course of an experimental PRV challenge [42]. For MRV, antibodies that can interfere with cell attachment, endosomal release or viral uncoating, i.e. antibodies against σ1, σ3, μ1c, and λ2, have been demonstrated to inhibit MRV infection in both cell culture systems and mice [21].

Potential explanations for why μNS and σ1 represent a preferential antigen combination in these DNA vaccines may be several. One hypothesis could be that the expression of μNS and formation of cellular inclusions induces a stronger innate immune response, leading to more efficient recruitment and activation of the adaptive immune cells, and that σ1 is an important antigen presented in MHC class I. This should lead to proliferation of cytotoxic CD8+ T-cells targeting cells presenting σ1 and μNS in a subsequent PRV infection. In this case, the cellular immune response play the main protective role. The gene expression data obtained here did not indicate that a stronger innate antiviral response was elicited in the presence of μNS expression, but rather that innate antiviral responses were triggered equally by the control plasmid, most likely due to pattern recognition receptors specific for nucleic acids. The adaptive immune gene expression indicated a non-specific trend towards higher CD8α and perforin expression in spleen for the vaccine expressing μNS and σ1. However, a significant increase in CD4 expression was observed only in the group expressing μNS and σ1. This point to MHC class II presentation and involvement of

the humoral arm of the immune system, which suggests a different mechanism: Overexpression of μNS may lead to cell death, which is cleared by macrophages. Macrophages phagocytosing antigen-containing cells or cell debris can present viral antigens in MHC class II, which are targeted by CD4+ T-cells, which in mammals has been shown to further support antibody production (sIgM) by B-cells [43]. This hypothesis better fits the previous finding of μNS-specific antibodies formed after PRV infection [42]. However, it was not a clear trend of increased expression of soluble IgM in our study. A reason for this could be that 6 weeks is a bit short for specific antibodies to develop, in line with previous observations [22,42]. In an earlier study, we found that SAV-based replicon vaccine induced an innate immune response with significant up-regulations of IFN-a in Atlantic salmon locally at the site of vaccination at 8 days post vaccination, induced primarily due to the replicon vector itself and not to the specific gene the replicon codes for [22].

Aquaculture confine animals under high density which generally facilitate transmission of infectious agents and reduced resistance to disease [44], and vaccination to control infectious diseases is necessary for the sustainability of aquaculture. PRV's ability to infect several species and its segmented genome prone to re-assortment, are factors that may ease rapid evolution. Control of PRV infection may therefore reduce a risk factor for the aquaculture industry, and the development of protective vaccine candidates against HSMI would be an important step.

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Conflict of interest

The authors declare that no financial or commercial conflict of interest exists in relation to the content of this article.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.10.094.

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