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Persistent immune responses in the heart determine the outcome of cardiomyopathy syndrome in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus (PMCV) is a severe cardiac disease in Atlantic salmon (*Salmo salar*) and one of the leading causes of morbidity and mortality in the Norwegian aquaculture industry. Previous research suggest a variation in individual susceptibility to develop severe disease, however the role of the immune response in determining individual outcome of CMS is poorly understood particularly in cases where fish are also challenged by stress. The present study's aim was therefore to characterize cardiac transcriptional responses to PMCV infection in Atlantic salmon responding to infection under stressful conditions with a high versus low degree of histopathological damage.

The study was performed as a large-scale controlled experiment of Atlantic salmon smolts from pre-challenge to 12 weeks post infection (wpi) with PMCV, during which fish were exposed to intermittent stressors. RNA sequencing (RNAseq) was used to compare the heart transcriptome of high responders (HR) with atrium histopathology score '4' and low responders (LR) with score '0.5' at 12 wpi. A high-throughput quantitative PCR (qPCR) analysis was used to compare immune gene transcription between individuals sampled at 6, 9 and 12 wpi. Based on RNAseq and qPCR results, RNAscope *in situ* hybridization (ISH) was performed for visualization of IFN-γ - and IFNb producing immune cells in affected heart tissue.

Compared to LR, the transcription of 1592 genes was increased in HR at 12 wpi. Of these genes, around. 40 % were immune-related, including various chemokines, key antiviral response molecules, and genes. associated with a Th1 pro-inflammatory immune response. Further, the qPCR analysis confirmed. increased immune gene transcription in HR at both 9 and 12 wpi, despite a decrease in PMCV. transcription between these time points. Interestingly, increased IFNb transcription in HR suggests the. presence of high-quantity IFN secreting cells in the hearts of these individuals. Indeed, RNAscope. confirmed the presence of IFN-γ and IFNb-positive cells in the heart ventricle of HR but not LR.

To conclude, our data indicate that in severe outcomes of PMCV infection various chemokines attract leucocytes to the salmon heart, including IFN-γ and IFNb-secreting cells, and that these cells play important roles in maintaining persistent antiviral responses and a sustained host immunopathology despite decreasing heart viral transcription.

1. Introduction

Cardiomyopathy syndrome (CMS), caused by piscine myocarditis virus (PMCV), remains a serious health issue in Atlantic salmon aquaculture. It has been estimated that as much as 20 % of all fish in affected sea cages are lost to the disease, amounting to an astounding one million individuals per year in Norway alone [1]. Of concern, CMS prevalence is increasing globally and has been detected in most salmon-farming areas including the Faroe Islands, Scotland, Ireland, and Canada [2].

Disease development is rather slow in PMCV- infected fish and CMS is typically diagnosed 1.5 years post-seawater transfer, but in recent years the outbreaks of CMS have been reported in post-smolt at 200–300 g size (i.e. shortly after seawater transfer) [3]. It is characterized by subendocardial inflammation and myocarditis, and in severe

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cases degeneration and necrosis of spongious myocardium [4]. Cardiac lesions first manifest in the atrium, progressing subsequently to the ventricular spongiosum. In advanced stages, inflammation evolve with massive lesions within these compartments. The compact myocardium is usually spared, but epicarditis (inflammation in the outer layer of the heart), is a common finding in this compartment [4].

Previous research demonstrated infiltration of mononuclear cells, including lymphocytes, monocytes and macrophages during the inflammatory process of the PMCV -infected heart [4]. In line with this, a study on late-stage disease (30–33 weeks post infection; wpi) revealed that ventricular CMS lesions consist of a mixture of T cells, IgM antibody-producing cells and MHCII + antigen-presenting cells, such as monocytes, macrophages, $CD83^{+}$ positive cells, and possibly granulocytes [5]. In advanced stages of CMS, profound subendocardial and myocardial cellular infiltration contribute to weakening of the cardiac atrium and sinus venosus walls, rendering them susceptible to fatal arrythmias and even atrial rupture during stressful interventions [6]. Moreover, CMS mortality seems to accumulate together with handling and stressful interventions in aquaculture operations [7], indicating that stress is a major factor not only for acute mortality but also in cardiac disease development. In addition, it is well known that the neuroendocrine stress system is intimately linked with the immune system [8].

Although PMCV infection is strongly implicated in the development of CMS, the pathogenesis of this syndrome remains unclear. In particular, there appears to be large inter-individual variation in the susceptibility to develop CMS, indicating variation in host resistance [9]. Interestingly, a previous study showed that experimental infections do not lead to severe pathology in all PMCV-infected fish [10,11]. That is, infected individuals diverged in two main directions; some individuals developed severe cardiac lesions (high responders; HR) and some individuals did not (low responders; LR) [11]. Furthermore, both viral load and severity of histopathological damage declined 12 wpi, consistent with the notion that other factors in addition to PMCV (e.g. stress) are necessary for development of severe disease and mortality [11]. However, the suggested mechanisms for the different outcomes and timeline of disease progression were based on a limited number of individuals and fish in these studies were not challenged by stressors commonly occurring under commercial farming conditions.

In mammals, individual variation in susceptibility to develop severe inflammatory cardiac disease is coupled to differential regulation of type 1 and type 2 immunity [12]. Type 1 immunity includes involvement of type 1 helper T cells (Th1) that secrete interferon-gamma (IFN-γ) to activate M1 macrophages and results in a proinflammatory response. Termination of inflammation depends on the switch to type 2 immunity that include contribution of type 2 helper T cells (Th2) which produce anti-inflammatory cytokines such as IL-4/IL-13 and M2 macrophage polarization that are associated with wound healing and tissue repair [12]. Whether different outcomes of PMCV infection can be linked to differential regulation of type 1 and type 2 immunity is unknown.

The aim of the present study was to characterize transcriptional responses to PMCV infection in HR versus LR Atlantic salmon experiencing stressful conditions, with a particular focus on immune gene transcription. We hypothesize that severe inflammatory disease in HR individuals develop due to persistent host-induced immune pathology.

2. Materials and methods

2.1. Fish, stress-stimulation, experimental challenge and sampling regime

The infection trial was performed at VESO Vikan (Namsos, Norway), a GLP-certified research station for infectious challenge experiments on aquatic organisms. The trial was approved by the Norwegian Food Safety Authority (Mattilsynet) through FOTS application ID 26555. Experimental design with election of sampling time points and PMCV inoculum was based on results from previous infection trials at the same research station [11]. A total of 300 unvaccinated Atlantic salmon (StofnFiskur Optimal strain) were obtained from the VESO Vikan Hatchery (Fosslandsosen) and distributed in three tanks at the VESO Vikan research station (Fig. 1). Each tank contained 100 fish with a mean ± SD weight of 38.3 ± 5.1 g at the onset of the experiment. Fish were reared at 12 ± 1 °C throughout the trial. Salinity was set to 0 ‰ pph and a 12h/12h light/dark photoperiod during acclimatization. This was then changed to 25 ± 2 ‰ pph salinity and 24h light photoperiod at vaccination and until the end of the experiment. Water flow was kept at 0.8 L/min and the maximum stocking density was 40 kg/m^3. Fish were fed 1 % of biomass per day by automatic feeders. When the fish reached a mean ± SD weight of 39.5 ± 4.8 g at experimental week 0, they were fitted with passive integrated transponder (PIT) tags. For this, fish were anesthetized in 200 mg/L Tricaine methanesulfonate (MS-222) before a PIT-tag was inserted by a cannula into the abdominal cavity. Fish were then returned to their home tank and monitored until recovery from anesthetic, and for the next couple of weeks, to control possible infections due to tagging. All fish recovered well and showed no signs of infection.

All fish were subjected to stressors during the experiment to mimic aquaculture conditions. During the freshwater period, fish were subjected to vaccination and one confinement stressor, followed by a non-lethal blood sample. That is, for vaccination (at experimental week 6), fish were randomly selected, anesthetized in 200 mg/L MS-222 and intraperitoneally injected with ALPHA JECT micro 6 (batch no. 451020, Pharmaq, Overhalla, Norway). Following this, a non-lethal blood sample (less than 1 % of body weight) was collected from the caudal vein with a 23G, 1 ml syringe containing the anticoagulant ethylene diamine tetra acetic acid (EDTA). The confinement stress challenge was conducted during experimental week 8, by lowering the water level in the holding tanks to 100 kg/m^3 for 40 min while aerating the water to maintain an oxygen level ≥65 %. Following this, a non-lethal blood sample was collected as described above. All blood samples were centrifuged at 4 °C and 650 rcf for 10 min and plasma was frozen at – 80 °C for later analysis.

At experimental week 12 (six weeks after vaccination), fish were challenged with PMCV by intraperitoneal injection of challenge isolate (0.1 mL, PMCV, j. no. 2828) following anesthesia in a bath containing 200 mg/L MS-222. Fish were starved for a minimum of 24 h prior to PMCV injection. At experimental week 17 (five weeks post infection; wpi) fish were again exposed to confinement stress, but no blood samples were collected at this timepoint.

In previous infection trials conducted at Veso Vikan [11], histopathological lesions associated with CMS were significant from 6 until 12 wpi. Based on the distribution of HR and LR to infection in these previous trials, we decided to collect samples at 6 (n = 54), 9 (n = 84) and 12 (n = 60) wpi to ensure a statistically robust sample size per contrast group at each sampling time point. For all samplings, fish were euthanized by an overdose of MS-222 (400 mg/L). Blood samples were centrifuged at 4 °C and 650 rcf for 10 min and plasma was frozen at – 80 °C for later analysis. Hearts were carefully excised, rinsed in PBS to remove blood and then processed in the following manner: a) Intact atria were fixated in 10 % buffered formalin b) Ventricles were cut in half horizontally and the lower half (including the ventricular apex) were fixated in 10 % buffered formalin for later histological and RNA-Scope analyses. c) the upper half (including the ventricular base) was stored in RNAlater (Invitrogen, Carlsbad, California, USA) stored at room temperature over night before storage at −20 °C.

2.2. Histopathology

Atrial and ventricular tissue from 196 fish was fixed in 10 % buffered formalin, processed in a Thermo Scientific Excelsior tissue processor (Thermo Scientific, Boston, MA, USA) and embedded in paraffin Histowax (Histolab, Askim, Sweden) using a Tissue–Tek, TEC 5 (Sakura, Alphen aan den Rijn, The Netherlands) embedding center. Embedded tissue was sectioned at 2 μm using a Leica RM 2255 Microtome (Leica

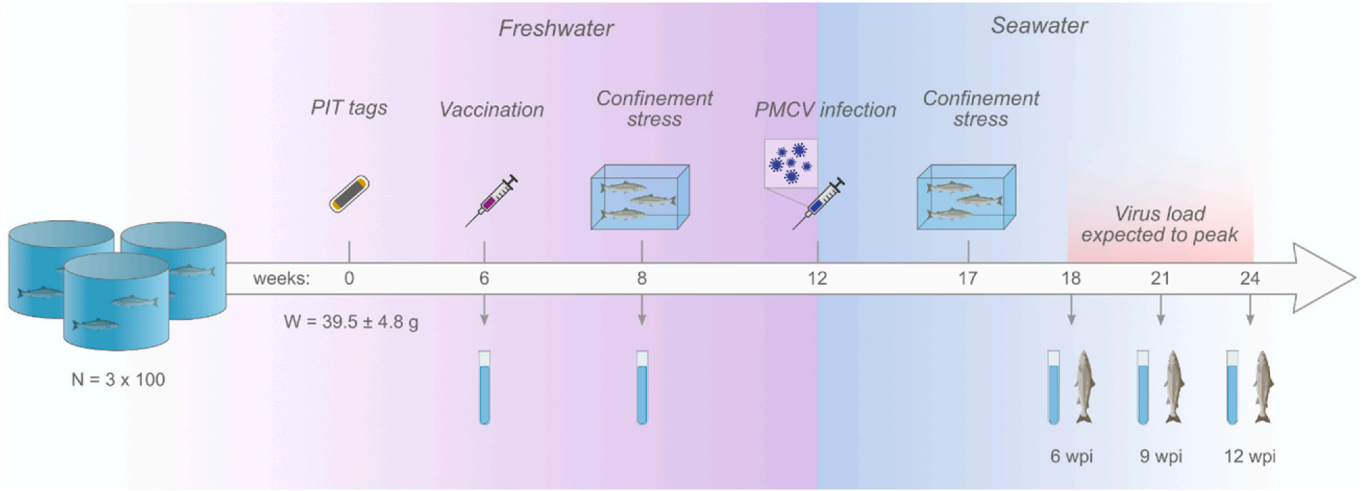

Fig. 1. Schematic overview of experimental fish, stress-stimulation, experimental challenge and sampling regime.

microsystems, Buffalo Grove, IL, USA), and stained with haematoxylin-eosin (HE). The stained slides were scanned in an Aperio GT-450 scanner and examined using Aperio ImageScope® (Leica microsystems). Spongious myocardium in the atrium and the heart ventricle was evaluated with respect to inflammatory lesions compatible with CMS. The inflammatory lesions were scored as follows: score 0 = no lesions, score 0.5 = <12.5 % of the myocardium affected, score 1 = 12,5–25 % of the myocardium affected, score 2 = 25–50 % of the myocardium affected, score 3 = 50–75 % of the myocardium affected and score 4 = >75 % of the myocardium affected. Based on the histopathological scoring of the atrium, individuals with scores 3 or 4 were classified as HR and individuals with scores 0, 0.5 or 1 as LR, in accordance with previous studies [11].

2.3. qPCR virus

RNA from heart tissue was isolated and prepared for qPCR analysis, as previously described [13].

RNA from head kidney and spleen samples was isolated using QIAcube (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentrations were measured with NanoDrop One (Thermofisher Scientific, Waltham, MA, USA). One microliter of the extracted RNA (100 ng/µl) was reverse-transcribed using the reverse transcription master mix (Fluidigm, San Francisco, CA, USA), according to the manufacturer protocol, and 1 µl cDNA was used in qPCR analysis based on protocol by Ref. [14]. The Ct values between different fish groups were compared by students T-test statistical analysis.

2.4. Cortisol plasma analysis

Cortisol in plasma from EDTA-treated blood was analyzed using DetectX® cortisol enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA), according to the manufacturers protocol. Briefly, plasma samples were pre-treated with dissociation reagent prior to dilution at in assay buffer at 1:100 final dilution. All standards, controls and samples were run in duplicates and read photometrically at 450 nm using a Multiskan™ FC spectrophotometer (Thermo Scientific™). Statistical analysis: cortisol data was analyzed using a linear mixed effect model (LME), with responder (LR vs. HR) and time (at vaccination vs. 2 weeks post-vaccination) as categorical independent variables and fish as a random effect. The initial LME models allowed the independent variables to interact. However, the final model was selected based on the lowest Akaike information criterion (AICc) score, i.e., the model with the best data fit when weighted against model complexity. Visual inspection of the qqnorm and residual plots to check the assumptions of normality and homoscedasticity confirmed that these models conformed to these assumptions. Interactive effects between treatment and test were assessed using Tukey–Kramer honestly significant difference post hoc test. Significance was assessed as $p \leq 0.05$.

2.5. Transcriptome sequencing

Five individuals from the HR group with histopathological score 4 and five individuals from the LR group with histopathological score 0.5 were used for RNA sequencing (RNAseq). The heart samples were cut into small pieces and placed in a 2 ml homogenizing tube containing two steal beads and 900 µl of the homogenization buffer (QIAzol Lysis Reagent, Sigma-Aldrich, Saint-Louis, MO, USA). The samples were then homogenized in a Precellys evolution touch homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5500 rpm for 20 s, followed by 30 s pause and a second homogenization round of 20 s. After this step, the RNA extraction was performed according to manufacturer's protocol for the QIAGEN RNeasy® Universal Mini Kit. (Qiagen). RNA concentration was measured using an Epoch microplate spectrometer (Biotek Instruments, Winooski, VT, USA) and calculated with the Gen 5 3.00 software (BioTek® Instruments, Inc). RNA integrity (RIN score) was quantified using the Agilent RNA 6000 Nano Kit according to manufacturer's protocol (Agilent, Santa Clara California, USA). RNA samples were kept at −80 °C until further analysis.

Sequencing of total RNA was completed by Novogene (Cambridge, United Kingdom). After additional quality testing at Novogene, total RNA samples were enriched with oligo (dT) magnetic beads for extraction of mRNA. First-strand cDNA was synthesized by randomly fragmenting the mRNA in fragmentation buffer, combining with random hexamers and assembling with M-MuLV reverse transcriptase. Complementary strands were then synthesized by nick translation using a custom (Illumina, San Diego, CA, USA) synthesis buffer containing dNTP's, RNase H and *Escherichia coli* polymerase I. The resultant cDNA library underwent adapter ligation, terminal repair, poly A-tailing, size selection and PCR enrichment, before a final quality assessment—concentration by Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), insert size by Agilent 2100 Bioanalyzer and quantification by qPCR. Libraries were sequenced as 150bp, paired end reads on an Illumina Hiseq 2500 instrument. For sequencing analysis, the samples for each group were compared to each other to identify common regulated genes.

2.6. Read mapping and quantification

All analyses in this section were completed on a Linux server using

command line operations. Quality metrics, such as phred quality score, duplicate reads and adapter contamination, were generated for each sample using Fastqc v0.11.8 and the sample qc reports collated using multiqc v1.9 [15]. As the sequence data had already been cleaned at the Novogene sequencing facility, quality across all bases was high (>30 phred score) and no adapters were present, so a quality trim was not required. Reads were aligned to the Atlantic salmon ICSASG_v2. (October 6, 2016) reference genome using HISAT2 v2.2.1 [16]. Reads that aligned to gene regions (as per genome feature definitions in ICSASG_v2 annotation) were counted using featurecounts v2.0.1 [17]. This count table formed the basis for gene expression analysis, which was performed using various R Statistical Software (v4.0.5: R Core Team 2022) packages.

2.7. Differential expression gene (DEG) analysis

To examine for batch effects and outliers, sample variance was estimated based on read count density, principal component analysis (PCA) and Euclidean distance, using base R functions 'density' and 'prcomp', and visualised using ggplot2 [18] and pheatmap (Pretty Heatmaps. R package version 1.0.12, 2019. Differential expression (DE) of genes between treatments was estimated with the DESeq2 package [19], which uses a negative binomial generalized linear model and shrinkage estimation of expression differences, designed to detect even small expression changes. Statistical significance ($p < 0.05$) of DE genes was estimated using a Wald test and then p values were adjusted for false-discovery using Benjamini-Hochberg [20].

2.8. Immune gene transcription analysis by high through-put qPCR

To better understand the regulation of immune genes of interest throughout the infection weeks (i.e. 6, 9 and 12 wpi), a high through-put PCR analysis of 96 target genes was conducted in 96 samples simultaneously (Biomark™ HD, Standard Biotools). The requirement for designing primers was described in previous research [21]. In addition to fifty genes previously used [21], key antiviral signaling molecules, antiviral effectors, pro-inflammatory and anti-inflammatory molecules (Th1 and Th2 response), cytokines, chemokines, markers for different leucocytes, apoptosis-related molecules, and a few uncharacterized proteins were included. The efficiency of all the primers have been tested and the list of genes and primers can be found in Supplementary Table 1.

RNA from salmon heart tissue was isolated by using QIAcube (Qiagen), according to the manufacturer's instructions. RNA concentration was measured with NanoDrop One (Thermofisher Scientific). One microliter of the extracted RNA (100 ng/μl) was reverse transcribed using the reverse transcription master mix (Fluidigm). Subsequently, the cDNA sample (1.25 μl) from each fish were mixed with the pooled 96 primer pairs (500 nM for each gene) and the PreAmp master mix (Fluidigm) and subjected to 12 pre-amplification cycles in a standard thermocycler (Thermo Fisher Scientific). The pre-amplified products were treated with exonuclease I (New England BioLabs, Ipswich, MA, USA) and mixed with SoFast EvaGreen supermix with Low ROX (Bio-Rad, Hercules, CA, USA) and 20 × DNA-binding dye sample loading reagent (Fludigm). The sample and primer mixes were transferred to the respective inlets of one 96.96 dynamic array IFC chip. The chip was primed in the BioMark IFC controller MX (Fluidigm) according to the Load mix 96.96 GE script. The loaded array chips were then placed in the BioMark HD system (Fluidigm) to proceed with the qPCR according to the GE 96 × 96 Fast PCR + Melt v2. pcl cycling program. Fluidigm RealTime PCR analysis software v. 3.0.2 was used to retrieve raw qPCR results, which were subsequently transferred to a relational database. The geometric means of three reference genes (ef1f, rps 20 and β-actin), which showed stability across samples, were used for calculation of ΔCt values. The ΔΔCt method was used to calculate the fold changes of gene transcription between HR and LR, and a Students T-test was used to check the significant changes (>1.75 folds, $p < 0.05$).

2.9. In situ hybridization (RNAscope)

RNAscope 2.5 HD Assay-red (Advanced Cell Diagnostics, Newark, CA, USA) was used for all *in situ* hybridization procedures following the manufacturer's guidelines [22]. All steps were conducted at room temperature, unless otherwise specified. In brief, 4 μm thick paraffin-embedded heart tissue sections from LR (n = 4) and HR (n = 5) were mounted on positively charged glass slides (Superfrost©; Mentzel, Thermo Scientific) and air-dried for a minimum of 24 h. These sections were then subjected to a 60 °C incubation for 90 min, followed by two rounds of 5 min treatment with xylene and two rounds of 1 min treatment with 100 % ethanol for dewaxing. Subsequently, the samples underwent a 10 min treatment with hydrogen peroxide to block endogenous peroxidase activity. The pretreatment included heat treatment in RNAscope® Target Retrieval Reagent at 100 °C for 15 min, followed by a protease treatment for 10 min at 40 °C to permeabilize the cells.

For hybridization, the slides were incubated with the target ZZ-probes for 2 h at 40 °C. Following hybridization, signal amplification was obtained by sequentially incubating the slides with the six amplification solutions provided in the assay kit. Signal detection was performed by incubating the slides with Fast Red chromogenic substrate for 10 min, and counterstaining was performed by immersing the slides in a 50 % Gill's haematoxylin solution for 2 min. Finally, the samples were mounted using EcoMount (BioCare Medical, Pacheco, CA, USA). All probes were designed and produced by the manufacturer based on user-provided sequences and have been catalogued and made commercially available. Details regarding the probes and positive and negative control probes including gene, target region, accession number and the manufacturer's catalogue number are available in Table 1.

3. Results

3.1. Histopathology

Individual fish were grouped into HR and LR contrast groups at 9 and 12 wpi based on a histopathological atrium score. Individuals receiving an atrium score of 3 (50–75 % of the myocardium affected) or 4 (>75 % of the myocardium affected) were categorized as HR, whereas LR received scores of 0 (no lesions), 0.5 (<12.5 % of the myocardium affected) or 1 (12,5–25 % of the myocardium affected) (Fig. 2). An overview of the number of fish in the HR and LR categories at the various sampling times is shown in Table 2. The overview of histopathological atrium score and PMCV transcripts in the heart samples is shown in Supplementary Fig. 1.

A B.

3.2. PMCV transcripts in heart and immune organs

PMCV abundance was investigated in hearts and immune organs at 6, 9 and 12 wpi. There was no significant difference in PMCV transcription in hearts of infected salmon with different histopathological scores at 6 wpi (Fig. 3). From 6 to 9 wpi, PMCV transcription increased

Table 1
Target and control probes for *in situ* hybridization.

	Probe	Accession no.	Target region (bp)	Catalogue no.
Target	IFNb	NM_001279095.1	7–552	1243801
	IFNg	AY795563.1	149–654	575871
Control	DapB (negative)	EF191515	414–862	310043
	PPIB (positive)	NM_001140870	20–934	494421

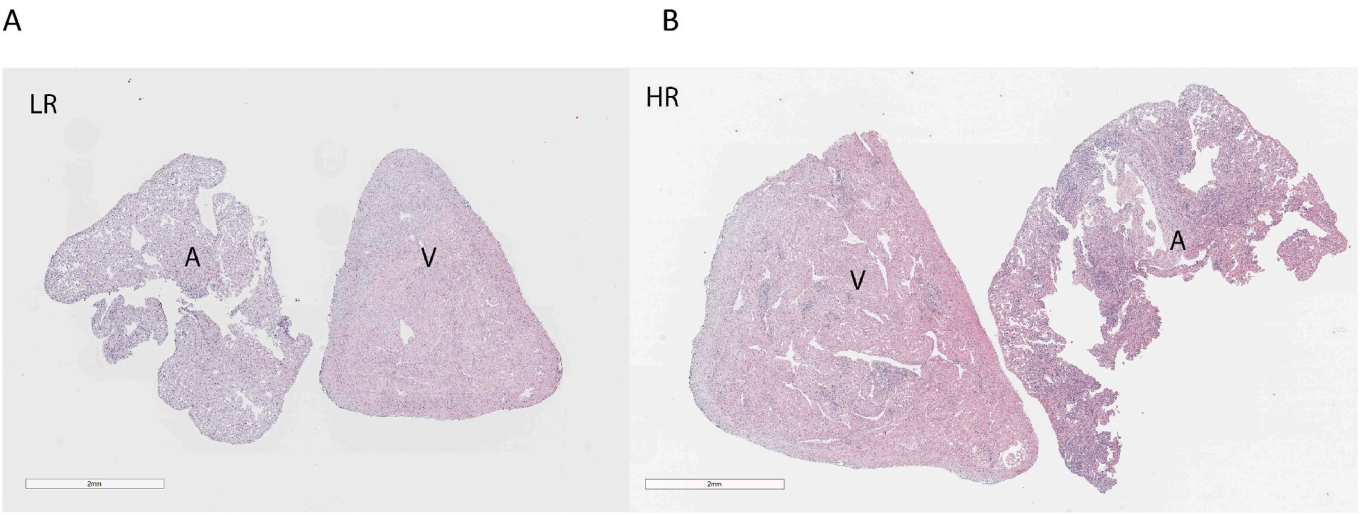

Fig. 2. Representative haematoxylin-eosin (HE)- stained cardiac sections from A) low (LR) – and B) high (HR) responding Atlantic salmon (*Salmo salar*) at 6 and 12 weeks post piscine myocarditis virus (PMCV) infection, respectively. In the LR heart there is multifocal mild inflammation (score 0,5) in both atrium (A) and ventricle (V). In the HR heart there is multifocal to diffuse inflammation in the atrium (score 4), and multifocal inflammation in the ventricle (score 2). Bar = 2 mm.

Table 2
Overview of the number of fish at 6, 9 and 12 weeks post-infection (wpi) with different histopathological atrium score used in different analyses.

WPI	Histo score atrium and contrast groups	*n*	% of all fish	Analyses
6 n = 54	Score 0	0	0	
	Score 0.5	13	24	5 fish for PCR
	Score 1	27	50	11 fish for PCR
	Score 2	14	26	8 fish for PCR
	Score 3	0	0	
	Score 4	0	0	
9 n = 83	**LR total**	**18**	22	
	LR (score 0)	0	0	
	LR (score 0.5)	5	6	4 fish for PCR
	LR (score 1)	13	16	11 fish for PCR
	Intermediate (score 2)	41	49	
	HR total	**25**	30	
	HR (score 3)	24	27	22 fish for PCR
	HR (score 4)	1	0	1 fish for PCR
12 n = 58	**LR total**	**13**	30	
	LR (score 0)	0	0	
	LR (score 0.5)	6	9	5 fish for RNAseq and *in situ* hybridization; 1 fish for PCR
	LR (score 1)	7	12	7 fish for PCR
	Intermediate (score 2)	21	36	
	HR total	**24**	41	
	HR (score 3)	19	33	19 fish for PCR
	HR (score 4)	5	9	5 fish for RNAseq and *in situ* hybridization

in HR, but not in LR hearts, and at 9 wpi the viral load was about 16 times higher in HR. The PMCV transcription decreased in LR and HR from 9 to 12 wpi. Still, viral load was 12.5 times higher in HR compared to LR at 12 wpi. Correlation of histopathology scores to viral load in hearts at 9 and 12 wpi is shown in Supplementary Fig. 1.

In head kidney and spleen, the highest PMCV transcripts occurred 6 wpi (Supplementary Fig. 2). At this time point, the PMCV transcripts showed a large variation within each fish group with the same pathology score and transcript abundance was not significantly different between fish groups. From week 6 to week 9, PMCV transcripts decreased significantly in head kidney and spleen of both HR and LR. From 9 to 12 wpi, the PMCV transcripts in head kidney or spleen of HR were generally twice of that in LR.

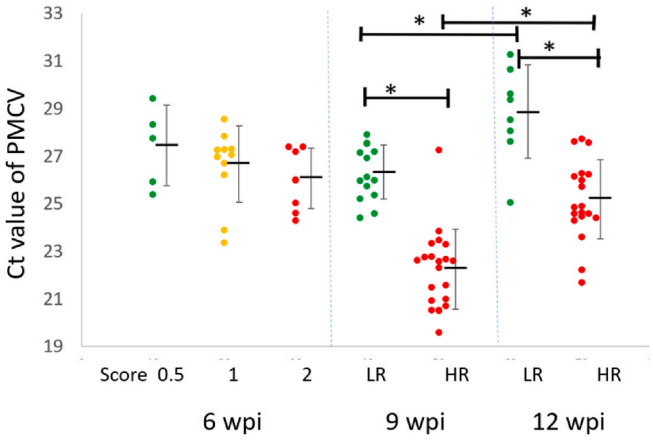

Fig. 3. Mean ± SEM Ct values of piscine myocarditis virus (PMCV) transcripts in hearts of low (LR, atrium histopathology score of 0, 0.5 or 1) – and high (HR, atrium histopathology score of 3 or 4) -responding Atlantic salmon (*Salmo salar*) 6-, 9- and 12-weeks post infection (wpi). Asterisks indicate significant differences ($p < 0.001$) between the compared two groups.

3.3. Plasma cortisol

Stressful challenges and the neuroendocrine stress hormone cortisol may impact the pathogenesis of immune pathologies like CMS. Thus, cortisol responsiveness was assessed in the two contrast groups to indicate potential differences in cortisol responsiveness. For this, non-lethal blood samples collected following two separate stressful interventions before infection were analyzed for plasma cortisol in retrospect. Samples were selected from fish categorized as HR and LR at 9 and 12 wpi. There was a significant effect of time ($\chi^2_{(1)} = 7.17, p = 0.007$) but not contrast group ($\chi^2_{(1)} = 0.58, p = 0.45$) on plasma cortisol levels after stress in LR and HR fish at the time of vaccination (vaccination stress) and 2 weeks after vaccination (confinement stress). Specifically, there were no significant differences in plasma cortisol concentrations between HR and LR at both timepoints, however, both HR and LR showed a decrease in plasma cortisol levels following vaccination compared to confinement two weeks later (p = 0.04 for both; Table 3).

Table 3
Mean (±SEM) plasma cortisol levels (in ng/ml) in low- (LR) *vs.* High-responders (HR) Atlantic salmon (*Salmo salar*) exposed to stressful challenges at vaccination and 2 weeks post-vaccination (wpi). Fish were categorized as LR and HR at 6 wpi and non-lethal blood samples were analyzed in retrospect from the same individuals. Small letters symbolize significant post hoc differences between groups.

Responder type	Timepoint	Plasma cortisol
LR	At vaccination	157 ± 10 [a]
HR	At vaccination	168 ± 12 [a]
LR	2 weeks post-vaccination	126 ± 14 [b]
HR	2 weeks post-vaccination	134 ± 11 [b]

3.4. RNA sequencing

Differential expression analysis was conducted on the most extreme contrast groups (LR with score 4, n = 5 *vs.* HR with score 0.5, n = 5, see Table 2) based on histopathological score at 12 weeks post infection. A total of 1661 genes were differentially expressed in the contrast groups, with 1592 upregulated genes and 69 downregulated genes in the HR compared to the LR group (Fig. 4 and Supplementary Table 2 for a complete overview of all differentially expressed genes).

Among all significantly DEGs, approximately 40 % were associated with immune responses. These included various genes encoding chemokines which were increased in HR (Fig. 5 and Supplementary Table 2).

A prominent feature of the transcriptome of the HR group was the widely increased transcription of interferon-induced antiviral genes and various tag-proteins (ISG15, SUMO, ubiquitin), ligases, as well as various pathogen pattern recognition receptors and SLAM family members. Furthermore, key regulators of the Th1 proinflammatory response, such as *IFNγ*, *Tbet* and spp*1* were also significantly increased in HR fish. Several interleukins, interleukin receptor subunits, markers for various leucocytes and cytotoxin molecules from CD8 T cells were also significantly increased in this group. For an overview of the top 40 DEG's see Supplementary Table 3, and the list of total DEG genes see Supplementary Table 2.

3.5. High-throughput qPCR (Biomark HD)

A high-throughput qPCR for immune gene transcription was performed on heart samples from 6, 9 and 12 wpi. At 9 wpi, immune gene transcription of 15 LR (atrium pathology score 0.5 and 1.0) was compared with 23 HR (score 3.0). At 12 wpi, data of 8 LR (score 1.0) was compared with 19 HR (score 3.0). For fish from 9 to 12 wpi, the severity of cardiac pathology was generally correlated with viral load, however, this correlation was not found in fish from 6 wpi (see Supplementary Fig. 1). Thus, for fish from 6 wpi, two sets of comparisons were performed: (1) a comparison of 8 individuals with atrium histopathology score '2' and 5 individuals with score '0.5'; (2) a comparison of individuals with higher *vs.* lower PMCV transcription (6 fish *vs.* 7 fish). At 6 wpi, only a few immune genes were differently expressed between fish with different histopathological scores. These included CD8a, IL8 and Carabin. When comparing individuals with higher or lower PMCV transcripts, a few genes (two *CCL19* chemokines, five antiviral effectors, *IL20Rα*, *SAA5*, *TNFRSF6B*, *CD274* and two uncharacterized proteins LOC106588383, LOC106588384) were significantly increased in fish with higher PMCV transcripts.

Increased immune gene transcription was found in HR at 9 wpi, including key antiviral signaling genes, antiviral effectors, genes associated with a Th1 pro-inflammatory response (*IFN gamma*, *IL8*, *IL18, SPP1*) and markers for various leukocytes (Fig. 6). Increased gene expression of markers of helper T cells (*CD4*), cytotoxic T cells (*CD8*), neutrophil granulocytes (*MPO*, *NCF1*, *SH3PXD2A*), dendritic-*like* cells (*CD83*), macrophages (*MARCO*), plasmacytoid dendritic-like cells (*IFNb*), antigen-presenting cells (*MHC II*) and B cells (*FCGR1A*) were present in the hearts of HR at 9 wpi. There were no significant differences between HR and LR with regards to the transcription of Th2-associated immune genes, as indicated by cytokines *IL4/13a*, *IL4/13b2*, *IL19*, receptor *IL13RA2* and transcriptional factor *GATA3*. The

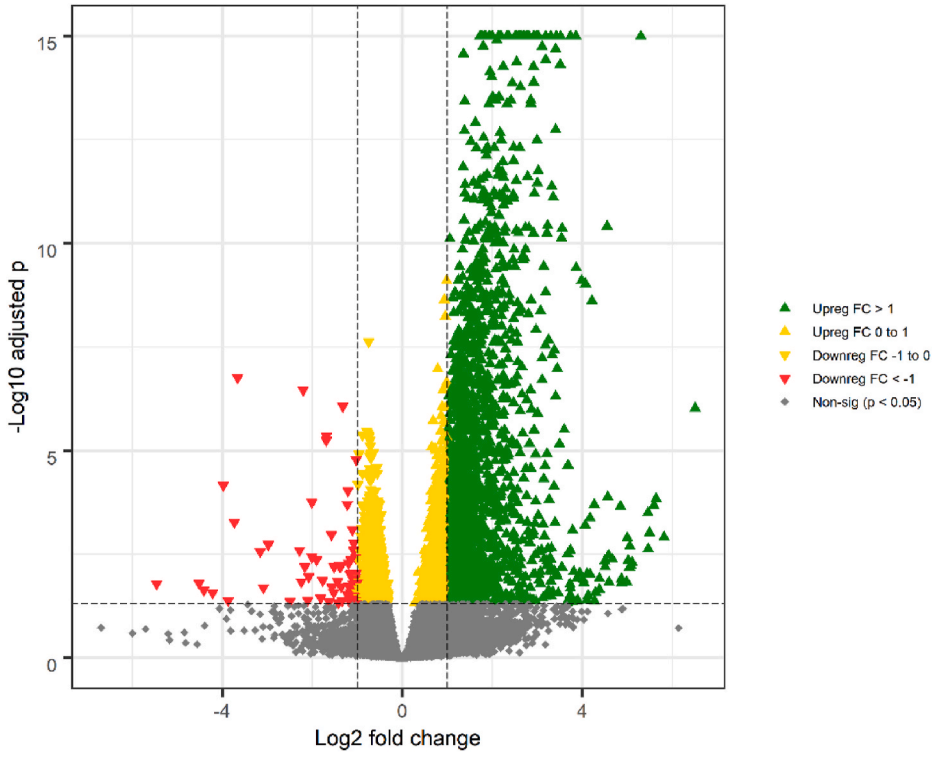

Fig. 4. Volcano plot of upregulated and downregulated differentially expressed genes (DEGs) in hearts of low (LR) - *vs.* high (HR) responding Atlantic salmon (*Salmo salar*) 12 weeks post infection with piscine myocarditis virus (PMCV). Statistics are based on the DESeq2 R package.

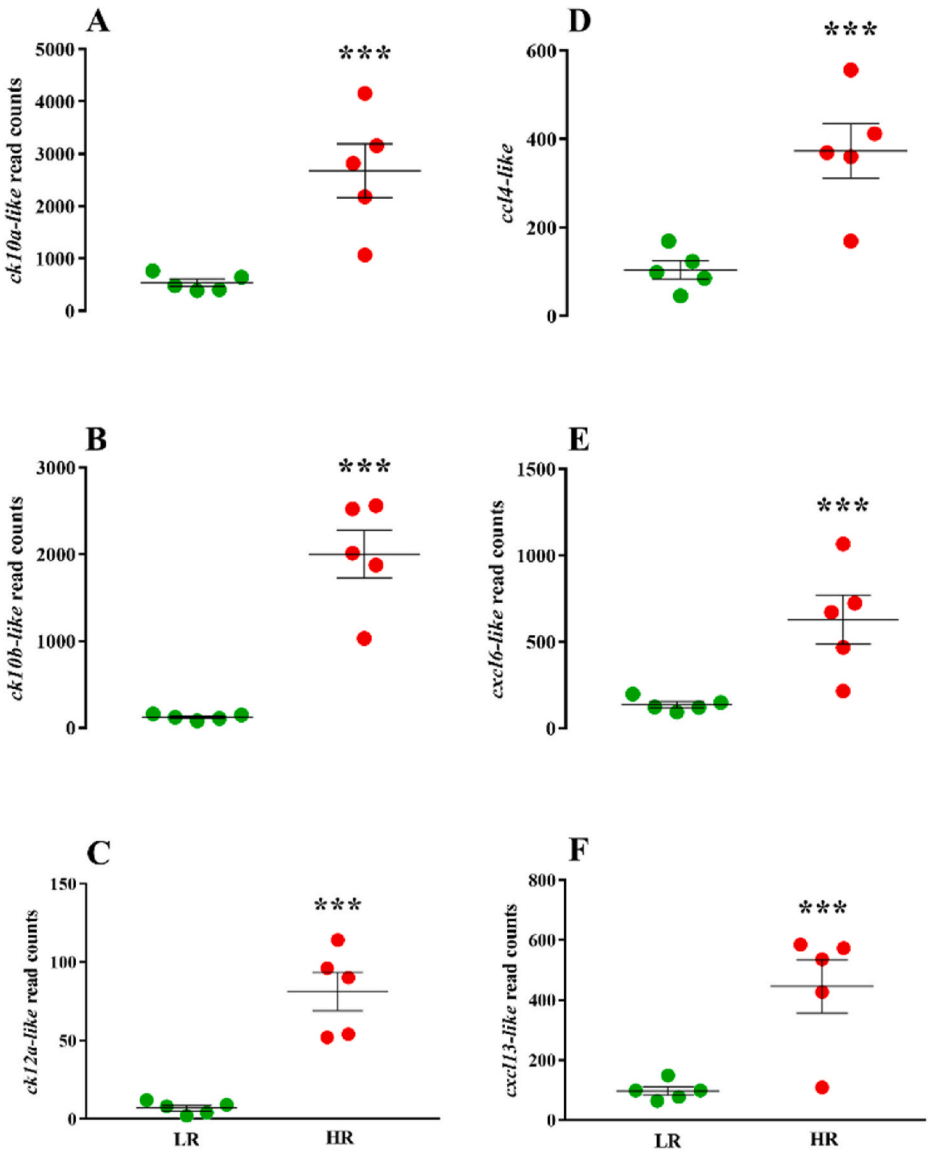

Fig. 5. Normalized read counts for A) chemokine CCL19 10a-like (ck10a-like), B) chemokine 10b-like (ck10b-like), C) chemokine 12a-like (ck12a-like), D) chemokine 4-like (ccl4-like), E) C-X-C motif chemokine 6-like (cxcl6-like) and F) C-X-C motif chemokine 13-like (cxcl13-like) in hearts of low (LR) - vs. high (HR)-responding Atlantic salmon (Salmo salar) at 12 weeks post infection (wpi) with piscine myocarditis virus (PMCV). Statistics are based on the DESeq2 R package.

anti-inflammatory cytokines *IL10a*, *IL10b*, *SCOS1* and related protein *IL10*RB were significantly increased in HR at 9 wpi. Among candidate genes for M2 macrophage markers (*TGFβ1*, *ARG1*, *ARG2*, *CXCR3-2* and *TGM2L)*, *TGFβ1* showed increased transcription in HR. All three subtypes of type I interferons (*IFNa*, *IFNb* and *IFNc*) showed significantly increased expression in HR at 9 wpi. At 12 wpi, *IFNb* was the only IFN subtype to be significantly increased in HR compared to LR.

3.6. *In situ hybridization (RNAscope)*

Based on results from RNAseq and qPCR, *in situ* hybridization (ISH) was conducted to visually depict the transcription of IFNb and IFNγ in affected heart tissue (Fig. 7). Among HR (histopathology score 4), IFNb-positive cells were identified in two of five individuals. These cells were scattered within the atrium, coexisting with other infiltrating leukocytes. Likewise, IFNγ-positive cells were observed in three out of five HR fish, exhibiting a similar scattered distribution pattern, with an additional detection of IFNγ-positive cells within the ventricle of one individual. Conversely, all LR fish (histopathology score 0.5) examined, were devoid of both IFNb and IFNγ-positive cells.

4. Discussion

In the present study we show that individuals responding to PMCV infection with a high degree of histopathological damage, are characterized by a persistent antiviral response and a sustained host immunopathology despite decreasing viral transcription. The origin and nature of the inflammation and tissue damage during CMS are largely unknown, however two likely causes of myocardial changes have been suggested: (1) damage caused by persistent viral replication and/or (2) host-induced immunopathology caused by infiltrated immune cells [11]. A correlation between viral transcripts and histopathological damage at 9 and 12 wpi in the present study (Supplementary Fig. 2) may at first glance indicate the first scenario. However, following an increase in cardiac viral loads at 9 wpi, PMCV transcript abundance decreased significantly at 12 wpi whereas the severe cardiac pathology persisted in the HR group. These findings suggest cardiac damage due to a persistent host-immune response during the later stages of the disease, rather than a persistent viral replication, and differ from findings of a previous study which demonstrated sustained or increased viral load with elevated pathology in HR [11].

Gene	W9	W12	Gene	W9	W12	Gene	W9	W12
Antiviral signalling and effectors								
IFNa			IRF7			ISG15		
IFNb			sCAS1			RSAD2		
IFNc			sCAS2			RTP2		
IFNg			TLR3			BANF1		
IRF1			TLR7			TRIB1		
IRF3			RIGI			SACS		
IRF5			MDA5					
Chemokines and markers for various leukocytes								
CK10a-CCL19			MHC I			ZNFX1		
CK10b-CCL19			MHC II			TAP1		
CCL4			CD4			CD53		
UCCL			CD8A			CD209		
CXCR3-2			CD28			CD83		
SELE			SASH3			CTLA4		
Cytokines, receptors and transcriptional regulators								
SPP1			IL8			IL4-13B2		
GATA3			IL18			IL13RA2		
SOCS1			IL19			IL10A		
TGB1			IL20RA			IL10b		
IL4-13A						IL10RB		
Molecules with known fuctions								
FERMT3			CAMP			ARG2		
HSP30			SAA5			ATF3		
HSPA5			LYZC2			C1QC		
MMP13			PLEKHF1			FCGR1A		
MMP9			MARCO			ADGRE5		
MPO			CH25H			CAL		
NCF1			CHIA			CRB		
SH3PXD2A			CAT			UMO		
LAO1			ARG1					
TNF and apoptosis								
TNFRSF14			CD40			CD274		
TNFRSF6B								
Uncharacterized proteins								
LOC106565512			TGM2L			LOC106588383		
LOC106588384								

Increased transcription (folds) indicated as:

1.3-1.9 1.9-2.8 2.8-4.0 >4.0

Fig. 6. Differentially expressed genes in heart ventricles of high (HR) - and low (LR)- responding Atlantic salmon (*Salmo salar*) 9- and 12-weeks post infection with piscine myocarditis virus (PMCV). Data are expression ratios (fold differences, HR-LR) and significant differences in magnitude of fold change are indicated as follows: yellow (1.3–1.9), pink (1.9–2.8), orange (2.8–4.0), red (>4).

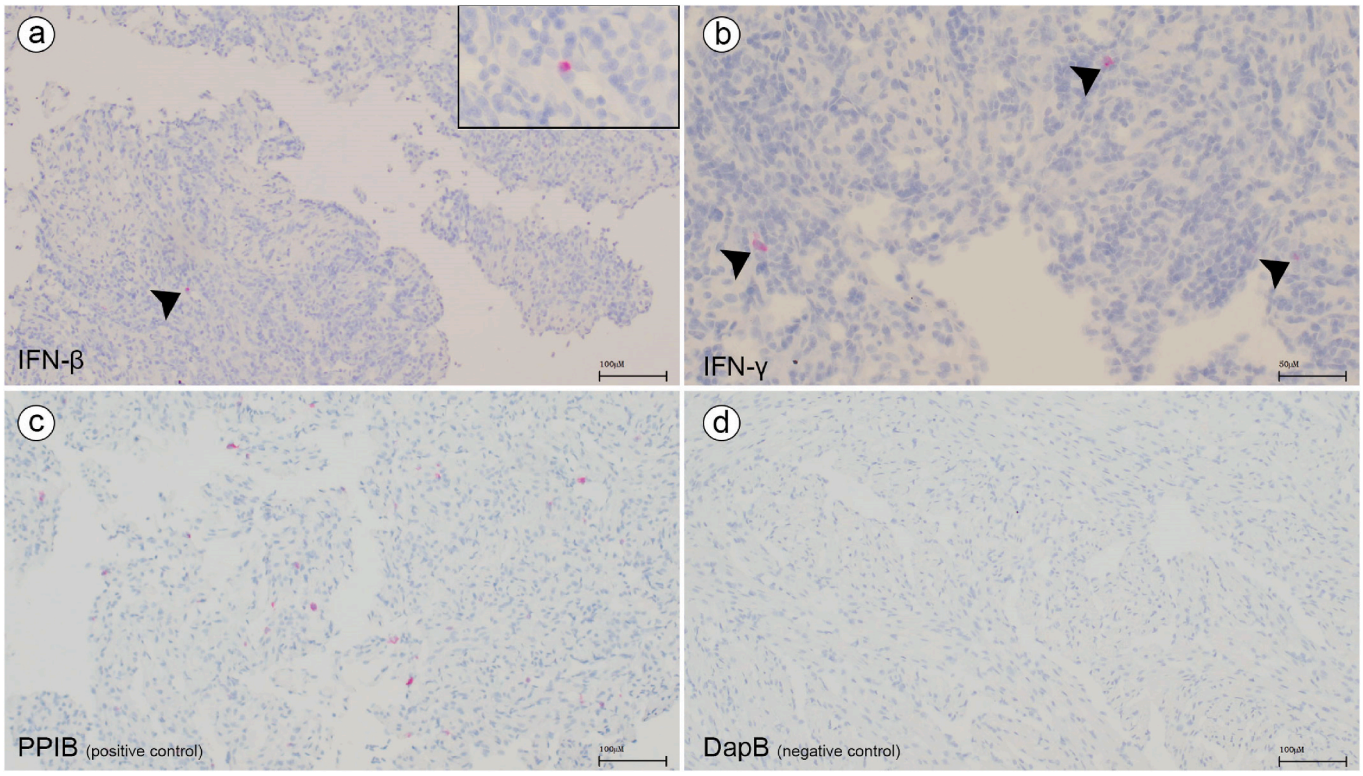

Fig. 7. *In situ* hybridization of IFNb (A) and IFNγ (B)-positive cells in heart tissue of high responding (HR) Atlantic salmon (*Salmo salar*) 12 weeks post infection with piscine myocarditis (PMCV) virus. a) A single IFNb-positive cell (arrowhead) in the atrium. Higher magnification in upper right corner. b) Scattered IFNγ-positive cells (arrowheads) in the atrium. c) Multiple PPIB-positive cells (red) in the ventricle (positive control probe). d) No detectable Dap-B-positive cells (negative control probe) in the ventricle.

An increased immune gene transcription in HR at 9 and 12 wpi support a scenario of a persistent immune response. Gene transcription indicated a Th1 pro-inflammatory signature of the adaptive immune response, which was significantly stronger in HR. The Th1 profile was evidenced by an increased transcription of key molecules like IFN-γ, Tbet, spp1 and IL-21, and was further supported by the presence of *IFN-γ* positive cells in the atrium and ventricle of fish from the HR but not the LR group. A Th1 response stimulate the development and accumulation of cytotoxic T cells which can directly kill virus-infected cells. Indeed, recruitment of T cells in HR hearts was indicated by increased expression of *CD8* and toxins such as perforin, granzyme A, granzyme K and stonustoxin in HR, in line with previous studies [10,11]. In parallel to having a role in the clearance of virus-infected cardiac cells, virus-specific cytotoxic $CD8^+$ cells may also contribute to an excessive tissue damage due to release of these pore-forming toxins, which may in part explain the severe cardiac lesion in HR although the PMCV transcripts decreased from 9 to 12 wpi. Macrophages exposed to inflammatory signals such as IFNγ, also typically assume a pro-inflammatory (M1) phenotype [23]. In humans, these M1 macrophages sustain cardiac inflammation by releasing inflammatory cytokines themselves and can also stimulate neighboring fibroblasts and cardiomyocytes into a pro-inflammatory state [24]. In contrast, M2 macrophages containing abundant arginase 2 (Arg2) may contribute to the subsequent recovery phase following viral clearance [25]. In the present study, we found no upregulation of Arg*2*, suggesting a sustained Th1 profile rather than Th2. It is interesting to note both the IL21 cytokine and the IL21R increased significantly in HR. IL-21 has potent regulatory effects on various leucocytes and is involved in suppressing the development of Th2 cells [26]. We found no differential expression of Th2 associated cytokine *IL4/IL13*-like molecule and transcription factor *GATA3* between HR and LR. Overall, our results indicate a Th1 pro-inflammatory profile in both LR and HR, which is particularly stronger in the latter group.

PMCV is not the only virus that causes heart disease in farmed salmon, as described by Yousaf et al. [6]. Other relevant viruses include Salmonid alphavirus (SAV) and Piscine orthoreovirus (PRV), the causative agents of pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI), respectively. Of relevance to the present study, a recent study demonstrated the presence of large numbers of M2 macrophages containing abundant arginase 2 in the heart of PRV-infected Atlantic salmon, which may contribute to the subsequent recovery following clearance of PRV-1 infection [25] and suggesting that the salmon heart has the ability to regenerate from virus-induced damage after PRV.

Early immune responses to viral heart infections including PMCV are associated with a marked upregulation of viral response genes, interferon (IFN) genes and IFN-dependent genes which usually level off during the course of the infection [10,11]. However, in addition to an early upregulation of antiviral effector genes, our results show a sustained antiviral and interferon response in HR also at later stages of the infection. In particular, we show an increased transcription of INFb in the HR group. In Atlantic salmon, three subtypes of IFN with confirmed antiviral activity have been identified, namely IFNa, IFNb and IFNc [27,28]. Upon stimulation of TLR7, mimicking a single-stranded RNA virus, the transcription of IFNb increased thousand folds [28]. According to previous research, salmon IFNb-producing cells may be similar to mammalian plasmacytoid dendritic cells, which are specialized high-quantity IFN-secreting cells [27]. IFNb-secreting cells appear absent from the heart in healthy fish, whereas a low number of cells reside in the head kidney and spleen [27]. The IFNb-secreting cells also produce IFNc [27], and both circulating IFNb and IFNc can induce the heart into an antiviral state, even without viral presence [29]. In the current study, a few IFNb + cells were found in the heart of HR but not LR fish. Although sporadic, these few but high-quantity IFNb-producing cells

may contribute to maintaining the heart in an antiviral state.

Increased transcription of various immune cell markers in HR hearts at 9 wpi, including helper T cells, cytotoxic T cells, B cells, neutrophil granulocytes, dendritic-*like* cells, macrophages and possibly plasmacytoid dendritic-like cells, suggest a marked immune cell traffic to the infected heart, and is in accordance with a previous study [5]. This immune cell recruitment was initiated by an increased transcription of several chemokines in HR, including six *CCL19* members. Chemokines of the CCL19 family are strongly expressed in lymphoid organs and tissues in mammals and induces migration of macrophages, T cells, and mature dendritic cells to lymphoid organs. In salmonids, *CCL19-CK13b* is highly expressed in thymus, head kidney and lymphoid tissue in the gill [30]. CCL19 (CK13a) increased significantly in the PRV and SAV-infected salmon heart [10], and recent research also confirmed that fish CCL19 could attract various immune cells, including macrophages that express high levels of IFN-γ [31]. It is interesting to note that *CCL4* (LOC106600447) shows significantly increased transcription in the HR group. CCL4 members were previously known as macrophage inflammatory protein (MIP-1β), but recent research confirmed their role in attracting various immune cells [32]. The CCL14-like chemokine (LOC106561115) has not previously been identified in salmon transcriptomes, and further research is needed to clarify its role in cell recruitment.

To examine the potential impact of stressors on CMS pathology development, all fish were exposed to stressors both before and after infection to simulate conditions in commercial aquaculture. Our results revealed comparable cortisol responses to stressors in both the LR and HR groups, suggesting that cortisol responsiveness might not be a primary factor contributing to the variability in individual responses to experimental PMCV infection. However, the disease progression observed in our study differed from prior research utilizing the same infection model. For example, in a study by Timmerhaus et al. [11] employing identical PMCV injection protocols and experimental facilities as in our current investigation, both viral load and histopathological damage peaked around week 8–9, followed by a decline in these parameters by 12 weeks post-infection. In contrast, none of the fish in our study were spared from cardiac lesions (i.e., scored 0), and while viral load decreased between weeks 9 and 12, histopathological damage persisted or even intensified at 12 wpi. Notably, the extent of histopathological damage in our study appeared greater, and the recovery from pathology seemed delayed compared to previous research. In aquaculture it appears that cumulative CMS mortality is associated with handling and stressful interventions. This implies that stress plays a pivotal role not only in acute mortality but also in the progression of CMS. Consequently, it is highly probable that the introduction of stress in conjunction with PMCV infection worsens the outcome of the infection. To validate this speculation, future studies should explicitly compare CMS development in stressed versus non-stressed individuals.

Interestingly, our results highlight two specific markers that could potentially be used for assessment of CMS severity. Firstly, the gene LOC106588384, encoding a membrane protein associated with a single nucleotide polymorphism on the trait loci on chromosomes 27, is strongly association with resistance to CMS [33,34]. Interestingly, LOC106588384 increased its transcription 22 times and was among the strongest increased genes in HR. Secondly, expression of LOC106561182 was increased in the HR group. This gene is a long non-coding RNAs (lncRNAs) which can be induced by orthomyxovirus and ISAV in several tissues [35,36]. Therefore, its tentative role as a biomarker for PMCV high responders should be further addressed.

In conclusion, we found that reduced virus loads from 9 to 12 wpi and persistent pro-inflammatory response in the hearts of HR fish implies that the severity of CMS may be more related to immunopathology caused by infiltrated leucocytes. In addition, the increase in PMCV transcripts was associated with a higher release of chemokines and cytotoxins, a stronger antiviral response, and a greater tendency to develop severe lesions, indicating negative effects of a prolonged type I immune response. We propose the use of two possible biomarkers to elucidate the severity of PMCV infection and further research should explore the feasibility of developing assays for these genes. Furthermore, future research is needed to clarify the detailed mechanism that results in persistent inflammation after PMCV infection in HR individuals.

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Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Baojian Sun: Conceptualization, Methodology, Study design, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Marco A. Vindas:** Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Simona Kavaliauskiene:** Investigation. **Håvard Bjørgen:** Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Erling Olaf Koppang:** Methodology, Study design, Supervision. **Helene Wisløff:** Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Michael Frisk:** Formal analysis, Investigation. **Hege Lund:** Conceptualization, Methodology, Study design, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Ida B. Johansen:** Conceptualization, Methodology, Study design, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109404.

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