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Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of *Piscine orthoreovirus*

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ABSTRACT

Piscine orthoreovirus (PRV) infection is common in aquaculture of salmonids. The three known PRV genotypes (PRV-1-3) have host species specificity and cause different diseases, but all infect and replicate in red blood cells (RBCs) in early infection phase. PRV-1 is the causative agent of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*), PRV-2 causes erythrocytic inclusion body syndrome (EIBS) in coho salmon (*Oncorhynchus kisutch*), while PRV-3 induces HSMI-like disease in farmed rainbow trout (*Oncorhynchus mykiss*). PRV-3 can also infect A. salmon without causing clinical disease and has been shown to cross-protect against PRV-1 infection and HSMI, while PRV-2 or inactivated adjuvanted PRV-1 vaccine only partially reduced HSMI pathologic changes. In the present work, we studied the transcriptional responses in blood cells of A. salmon two- and five-weeks post infection with PRV-1, PRV-2, PRV-3, or post injection with inactivated PRV-1 vaccine. PRV-1 and PRV-3 replicated well in A. salmon blood cells, and both induced the typical innate antiviral responses triggered by dsRNA viruses. Two weeks post infection, PRV-3 triggered stronger antiviral responses than PRV-1, despite their similar viral RNA replication levels, but after five weeks the induced responses were close to equal. PRV-2 and the InPRV-1 vaccine did not trigger the same typical antiviral responses as the replicating PRV-1 and PRV-3 genotypes, but induced genes involved in membrane trafficking and signaling pathways that may regulate physiological functions. These findings propose that the protection mediated by PRV-3 against a secondary infection by PRV-1 occur due to a potent and early activation of the same type of innate immune responses. The difference in the timing of antiviral responses may give PRV-1 an evolutionary edge, facilitating its dissemination to A. salmon heart, a critical step for HSMI development.

1. Introduction

Unlike mammals, teleost red blood cells (RBCs) are nucleated and possess an active transcriptional/translational machinery essential for gene expression [1,2]. *Piscine orthoreovirus* (PRV, family *Spinareoviridae*, genus *Orthoreovirus*), a virus with an icosahedral, double-layered capsid, and a segmented double-stranded RNA genome [3,4], replicates in salmonid RBCs [5,6]. PRV infection is associated with disorders of the circulatory system and is a significant threat in salmonid aquaculture [7]. There are three genotypes of the virus, PRV-1, PRV-2 and PRV-3, which demonstrate similar systemic dissemination but different pathogenicity in different salmonid species [8]. PRV-1 primarily targets farmed Atlantic salmon (*Salmo salar*) and causes heart and skeletal muscle inflammation [9], a prevalent viral disease in A. salmon aquaculture in Northern Europe [7,10–12]. PRV-1 establishes a persistent infection, and is ubiquitous in the marine phase of farmed A. salmon [13]. Genetic viral reassortants have different virulence, and PRV-1 is also commonly detected in fish populations without clinical signs of disease [14,15]. PRV-3 was first detected in Norway in 2013, in farmed rainbow trout (*Oncorhynchus mykiss*) with pathological lesions resembling HSMI [16]. A causative role of PRV-3 in heart inflammation in rainbow trout was experimentally confirmed in 2019 [17]. PRV-1 and PRV-3 genotypes have also been found and associated with jaundice syndrome in Chinook salmon (*Onchorynchus tshawytscha*) in British

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Columbia and coho salmon (*Oncorhynchus kisutch*) in Chile [18,19]. PRV-2 is the etiological agent of erythrocytic inclusion body syndrome (EIBS) in Japanese coho salmon aquaculture [20] and has been found in wild coho salmon in Alaska [20,21].

Previous transcriptional analyses of A. salmon RBCs have revealed expression of a wide repertoire of pattern recognition receptors (PRRs), interferon (IFN) transcription regulators and IFN inducible genes known to confer resistance to viral infections, several of which were strongly activated in response to *ex vivo* and *in vivo* exposure to PRV [2,22,23]. The PRV virion, akin to the infection mechanism described in mammalian orthoreovirus (MRV), is internalized into the host cell via receptor-mediated endocytosis. The outer capsid proteins undergo proteolytic degradation, facilitating penetration of viral core particles across the late endosomes [9,24]. Although the membrane proteins implicated in PRV internalization remain unknown, viral recognition upon entry into host cells has been associated with the endosomal toll like receptor 3 (TLR3) and potentially ATP- dependent RNA helicase DHX58 (also referred to as retinoid acid-inducible (RIG)- like receptor 3) [22,23]. In the cytoplasm, the viral assembly occur in globular neo-organelles, referred to as viral factories, which provide an environment conducive to viral replication, potentially evading detection by host cell innate immune system [9,24,25]. The peak of PRV infection in A. salmon RBCs typically occurs between two to five weeks post exposure, coinciding with high plasma viremia and antiviral responses [9,22]. Both humoral and cellular responses are elicited, leading to infiltration of immune blood cells into the heart and production of PRV- specific antibodies [26–28].

Despite the significant impact of PRV infection on salmonid aquaculture, the absence of robust monitoring strategies and effective prevention measures remain a pressing concern [7,29]. Experimental vaccines against HSMI have been developed and tested, including an inactivated whole virus vaccine based on virulent PRV-1 [30], and DNA vaccines encoding PRV non-structural proteins [31]. These vaccines only led to partial protection against HSMI. Recently, PRV-3 infection in A. salmon was shown to efficiently block consecutive PRV-1 infection and HSMI. In comparison, the injection of PRV-2 and inactivated adjuvanted PRV-1 vaccine did not protect from infection, and only partially reduced HSMI pathology. Only PRV-3 triggered PRV-1 specific antibody production [29], as demonstrated using a bead-based immunoassay [27]. Given the importance of developing effective vaccines in salmonid aquaculture against PRV-1 infection, understanding the link between initial responses in infected blood cells and the effective cross protecting potential of PRV-3 are of great interest. In the present study, we report on transcriptional differences and similarities in whole blood of A. salmon infected with PRV-1, PRV-2 and PRV-3 two and five weeks post-injection. We focused on factors that may be involved in the previously reported cross-protection mediated by PRV-3, but not PRV-2, and early responses that may explain why PRV-1 infection leads to a pathological outcome in A. salmon, while PRV-3 does not.

2. Materials and methods

2.1. Experimental trial and blood sampling

Blood samples from A. salmon infected by either PRV-1, PRV-2 or PRV-3, immunized with an inactivated, adjuvanted PRV vaccine (InPRV-1), and mock controls originated from a previously published experimental trial [29]. Briefly, 300 fish of a mean weight of 41.3 g (± 5.8 g) were divided into five experimental groups and kept in freshwater (10 °C, 24:0 light: dark cycle, >90 % O2). The experimental fish were injected intraperitoneally (IP) with 0.2 mL of the following materials. The PRV-1 infection material was based on an infected blood pellet (PRV-1 isolate NOR2012-V3621). The isolate had been passaged in previous experimental trials in A. salmon, all resulting in HSMI [9]. The PRV-3 infection material was prepared from a blood pellet harvested during a disease outbreak in 2014 (PRV-3 NOR2014) [16], that had been passaged in rainbow trout leading to HSMI-like pathology [17]. Mock blood lysate was obtained from non-infected A. salmon. Frozen blood pellets from PRV-1, PRV-3 and mock control samples were diluted 1:10 in L15-medium, sonicated, centrifuged, and the supernatant was collected. PRV-2 infection material originated from a frozen spleen sample from coho salmon [20]. The tissue sample was homogenized in L15 medium, sonicated and centrifuged. Inactivated PRV-1 material was prepared from purified PRV-1 particles (PRV-1 NOR2012, $5.35 \bullet 10^9$ copies/mL) by PHARMAQ AS described earlier [30]. Briefly, the batch was inactivated by formalin immersion and prepared as a water-in-oil formulation where the water phase (containing PRV antigens) was dispersed into a mineral oil continuous phase containing emulsifiers and stabilizers. Ten weeks post injection with PRV-1-3 infection material or InPRV-1 vaccine, the groups were infected horizontally by addition of PRV-1 infected shedder fish, and the full immunization trial lasted 18 weeks, reporting on the cross-protective potential of the injected viruses and inactivated vaccine against subsequent PRV-1 infection and HSMI [29]. Additional details on the trial are presented in Malik & Teige, 2021 [29].

Eight fish were sampled prior to injection (week 0), and from each of the five experimental groups (PRV-1-, PRV-2- or PRV-3- infected fish, immunized fish with InPRV-1 vaccine and mock controls) at week 2 and 5 after IP injection. Blood was drawn from the caudal vein of the fish using BD Medical Vacutainer heparin-coated tubes (BD Medical, Mississauga, ON, USA). The samples were stored at 4 °C for a maximum of 6 h, centrifuged (3000×*g* for 5 min at 4 °C), and plasma and blood pellets were separated into different microtubes and stored at −80 °C.

In the present study, blood samples from six fish per group sampled at week 2 and four fish per group sampled at week 5 were analyzed. In addition, blood samples from four fish sampled at week 0 were used as additional controls. The sample selection was based on RNA quality, to ensure optimal RNA-seq results.

2.2. RNA isolation and sequencing

Blood cell pellets of 20 μL were resuspended in MagNA Pure LC RNA Isolation Tissue (Roche) to a final volume of 400 μL and homogenized using 5 mm steel beads and TissueLyzer for 3 min at 25 Hz. MagNA Pure 96 Cellular RNA Large Volume Kit (Roche) was used for automated total RNA isolation following the manufacturer's protocol. RNA was quantified using Multiskan SkyHigh microplate spectrophotometer (Thermo Fiscer Scientific). RNA quality (RIN >8) was ensured using Agilent 2100 Bioanalyser (Agilent, USA) before being sent for sequencing.

Total RNA from 30 samples harvested week 2 (Mock control, n = 6; PRV-1 infected, n = 6, PRV-2 infected, n = 6; PRV-3 infected, n = 6; Inactivated PRV-1, n = 6), 20 samples harvested week 5 (Mock control, n = 4; PRV-1 infected, n = 4, PRV-2 infected, n = 4; PRV-3 infected, n = 4; Inactivated PRV-1, n = 4), and 4 samples from week 0, were sent to the Norwegian Sequencing Center (NSC, Norway). Library preparation was performed using strand-specific TruSeq mRNA-seq Library prep kit (Illumina, CA, USA). The libraries were pooled and sequenced on one lane of Illumina NovaSeq S4 flow cell to obtain 150bp paired end reads. The raw sequencing data are available in NCBI SRA BioProject - PRJNA1148351.

2.3. RT-qPCR for PRV variants

RNA loads of PRV-1 and PRV-3 were assessed using Qiagen One-Step RT-qPCR kit (Qiagen). The input was standardized to 50 ng (10 μL of 5 ng/μL) of total RNA per reaction and the samples were run in duplicates. Prior to RT-qPCR, the template was denatured at 95 °C for 5 min. The RT-qPCR reactions were performed under the following thermal conditions: 50 °C for 30 min, 94 °C for 15 min, and 45 cycles for 30 s at 95 °C and 1 min at 60 °C. To define a sample as positive, a cutoff of Ct < 35 was set. For PRV-2, a Quantitect SYBR Green RT-qPCR kit (Qiagen) was used according to manufacturer's instructions. A total of 50 ng RNA (5 μL of

10 ng/μL) was denatured at 95 °C for 5 min and the samples were run in duplicates with the following thermal conditions: 50 °C for 30 min, 94 °C for 15 min, and 40 cycles for 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. To assess the specificity of the assay, melting curve analysis was performed. A cutoff of Ct < 35 was set, similar for PRV-1 and PRV-3. Probes and primer sequences are given in Supplementary File A, Table A1 [29].

2.4. Bioinformatic processing and statistical analysis

Raw sequence data (Fastq files) were processed to trim/remove adapter and low quality sequences using BBDuk tool in BBMap v.38.18 suite (parameters: ktrim = r, k = 23, mink = 11, hdist = 1, tbo, tpe, qtrim = r, trimq = 15, maq = 15, minlen = 36, forcetrimright = 149) [32]. Cleaned reads were mapped to *Salmo salar* genome (ENSEMBL ICSASG_v2) using the HISAT2 v.2.2.1 (parameters: rna-strandness RF) [33]. FeatureCounts v.1.4.6-p1 (parameters: p -s 2) was used for estimating the number of reads and aligning against the reference genes in ENSEMBL r104 GTF annotation [34]. Initial raw data analysis was performed using SARTools v.1.7.4 and R v.4.1.1 [35,36]. Normalization and differential expression between groups and against the control at week 2 and 5 were performed using DESeq2 v.1.34.0 [37]. The annotation tables were cleaned using median count reads >10 as a cut off, to omit genes with zero or low counts. Adjusted p-value (padj) was calculated using Benjamin-Hochberg (BH) correction and gene with padj below 0.05 were considered as differentially expressed genes (DEGs). For gene regulation, upregulated features with less than 2-fold change and downregulated features with higher than 0.5- fold change in expression (0.5 < fold change <2) were filtered out.

STRING Database v.12.0 was used for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with 0.05 as p-value cutoff, BH adjusted [38]. In particular, DEGs were sorted into functional categories via KEGG pathway and gene ontology GO enrichment analyses. Potential functions of uncategorized genes were explored within databases primarily focused on mammalian genome and gene function, such as Reactome and InterPro, and were also considered. The magnitude of transcriptional similarities/differences of each PRV genotype to PRV-1 was determined using heatmaps. To better understand how PRV immunization/injection affected gene regulation over time, heatmaps were constructed using Log2-fold changes (Log2FC) of a selected set of DEGs, compared to week zero (transcripts) as baseline reference.

3. Results

3.1. Overview of PRV immunization trial data

The transcriptional analysis performed in this study further investigates key observations from a previous published work by Malik & Teige et al., 2021 [29]. Here, we measured the RNA load of PRV

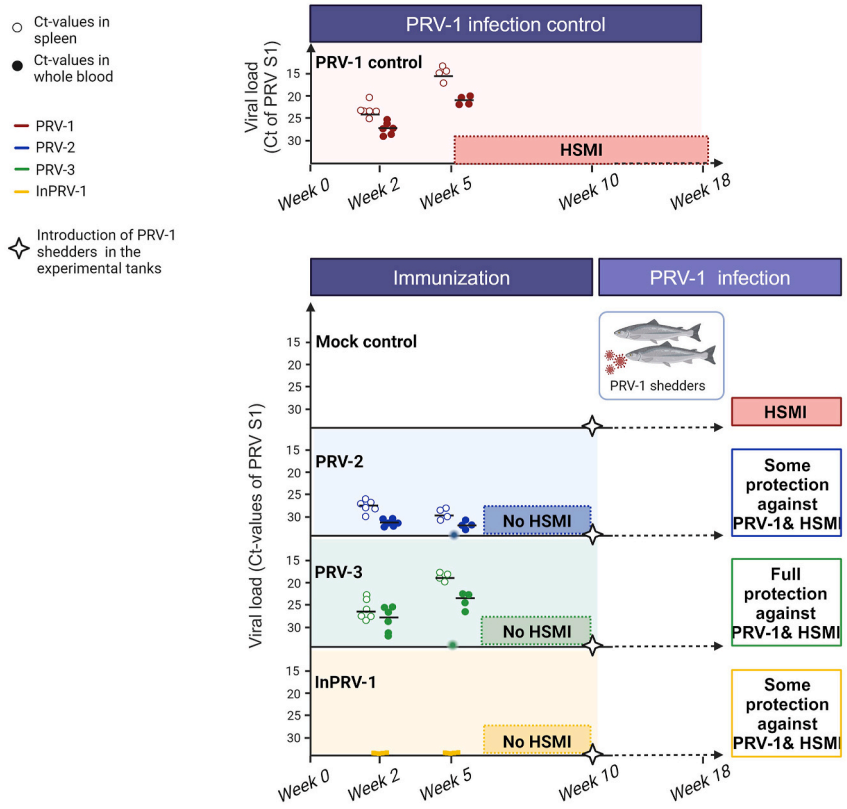

Fig. 1. Experimental setup and results of the original Piscine orthoreovirus (PRV) infection trial [29]. Fish were allocated into five experimental groups injected intraperitoneally (IP) with blood lysate containing PRV-1 or PRV-3, spleen homogenate containing PRV-2, purified, inactivated and adjuvanted PRV-1 (InPRV-1 vaccine control) and blood lysate originating from uninfected healthy fish (Uninfected control). RNA loads of PRV-1-3 in spleen (open dots) and whole blood (filled dots) were measured two- and five-weeks post injection using RT-qPCR assays targeting virus-specific parts of the S1 genome segment. Virus levels are presented as Ct-values for each individual and as average (n = 6/group in week two and n = 4/group in week five). PRV-1 levels colored red; PRV-2 levels colored blue; PRV-3 levels colored green; InPRV-1 colored yellow. A PRV-1 cohabitation challenge took place 10 weeks post immunization, in the PRV-2-, PRV-3-infected groups and InPRV-1 vaccinated controls, along with the uninfected control group. The infection outcome and cross-protection conferred by the three PRV genotypes and InPRV-1 vaccine was assessed through histopathological analysis of HSMI in week 15 and 18, as mean of 8 individuals (score 0- no HSMI, score 3- full HSMI) by Malik and Teige [29]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genotypes in whole blood of A. salmon two and five weeks post injection, to explore potential correlation between transcriptional responses and viral replication status. These results are shown in Fig. 1, together with an overview of the original experimental setup and key findings reported by Malik & Teige et al., 2021 [29]. The trial consisted of two distinct parts; fish immunization (week 0–10) and secondary PRV-1 infection by cohabitation challenge (week 10–18). RNA loads of PRV genotypes in spleen were similar in week 2 (~Ct 25), but diverged in week 5, where PRV-1 and PRV-3 levels increased, while PRV-2 levels decreased over time [29]. In whole blood, RNA loads of PRV-1 and PRV-3 showed the same increasing pattern over time as in spleen, whereas PRV-2 levels were lower both week two and five. These findings

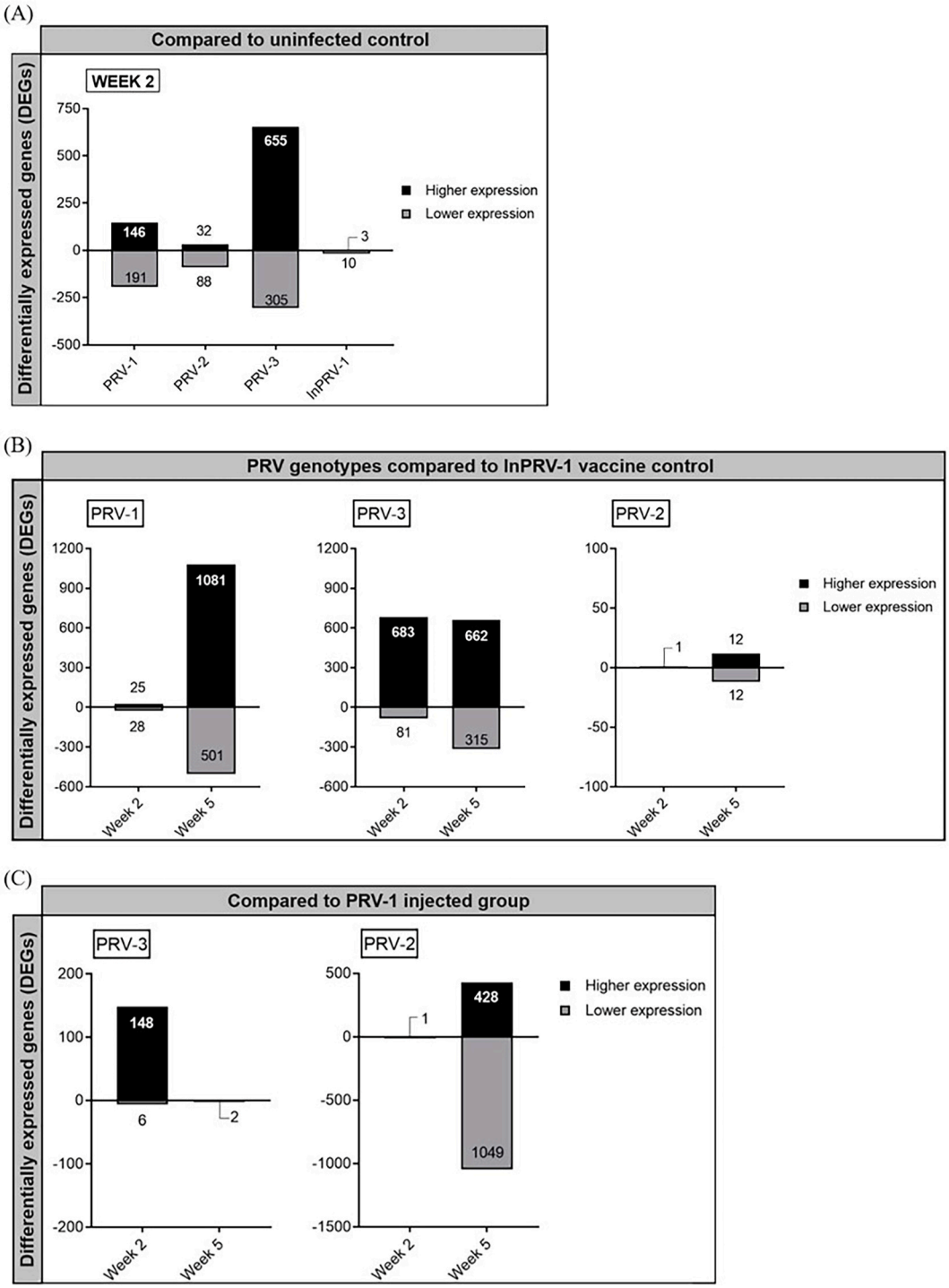

Fig. 2. Number of significantly regulated genes (DEGs) in whole blood of A. salmon infected by PRV genotypes and the inactivated adjuvanted PRV-1 (InPRV-1) vaccinated control. (A) Number of DEGs in whole blood of A. salmon two weeks after infection, compared to uninfected controls. Cutoff ≥ 2- fold (higher) and ≤0.5- fold (lower), and a cutoff of normalized median read counts ≥10 were applied. (B) Number of DEGs in whole blood of A. salmon two and five weeks after infection compared to vaccinated with vaccinated controls. Cutoff ≥ 2- fold (higher) and ≤0.5- fold (lower), and a cutoff of normalized median read counts ≥10 were applied. (C) Number of DEGs in whole blood of A. salmon two and five weeks after infection compared to PRV-1 infected fish. Cutoff ≥ 2- fold (higher) and ≤0.5- fold (lower), and a cutoff of normalized median read counts ≥10 were applied.

supported the ability of PRV-2 and PRV-3 to infect A. salmon when injected IP. However, in contrast to the original analyses in spleen, only PRV-1 and PRV-3 could be confirmed to replicate in whole blood.

The protection against secondary PRV-1 cohabitation challenge (week 10), and HSMI was shown by histopathological analysis of heart tissue at week fifteen and eighteen [29]. Infection by PRV-3 efficiently blocked secondary PRV-1 infection and HSMI (no individual developed pathology, HSMI mean score = 0), while PRV-2 and InPRV-1 injection only partially protected against HSMI (PRV-2: 6 out of 8 fish, HSMI mean score = 2, and InPRV-1: 2 out of 8 individuals, HSMI mean score = 0,5) [29].

3.2. Transcriptional analysis of infected and uninfected Atlantic salmon whole blood

In the RNA-Seq data obtained from blood, the reads were mapped to a total of 55,819 features (genes) in the A. salmon genome (ENSEMBL ICSASG_v2/ENSEMBL r104 annotation). Information on total sequenced reads and alignment rate of mapping of the biological groups in week zero, two and five is provided in Supplementary File B, Tables A1 and A2. Most of the samples showed overall alignment rate >75 %, to the A. salmon genome.

Principal component analysis (PCA) was performed to assess the variability of samples from infected groups and uninfected controls at two and five weeks post injection (Supplementary File B, Figs. A1 and A2). All biological groups showed wide dispersion at week two, but blood replicates from PRV-1 and PRV-2- infected fish tended to cluster in closer proximity. PCA at week five showed lower variability within the biological replicates of each infected group, while distribution of the clusters against the first principal component indicated that PRV-1 and PRV-3, along with PRV-2 and InPRV-1 are more closely related at week 5. Considering the increasing viral load of PRV-1 and PRV-3 from week 2–5, not found for PRV-2 and InPRV-1 in whole blood (Fig. 1), PCA clustering may be in line with the replication status of each PRV genotype.

Two uninfected controls from week two were identified as outliers in the PCA plot. Three out of four controls from week five showed an overall alignment rate below 50 %. Therefore, these samples were omitted from further analysis.

3.3. Differentially expressed genes in whole blood of PRV- infected Atlantic salmon

Differential gene expression analysis was performed to assess differences in gene expression patterns between immunization groups (PRV-1, 2, 3 and InPRV-1) and compared to uninfected controls of whole blood of A. salmon at week two (Fig. 2A). Whole blood of PRV-3 infected fish showed the greatest transcriptional differences compared to uninfected controls (655 genes with higher expression and 305 with lower expression level). PRV-1 triggered intermediate transcriptional differences, with more genes showing lower (191 genes) than higher (146 genes) expression compared to controls. Immunization with PRV-2 resulted in the fewest transcriptional differences out of the three PRV genotypes (88 higher and 32 lower expressed genes). For PRV-1 vaccine group, there were almost no expression differences compared to the uninfected controls. No such comparison was performed between infected and uninfected groups at week five, as week five control datasets were excluded.

To identify shared and/or unique expression patterns, we compared the whole blood transcriptional responses of all PRV- infected groups to each other both for week two and five (Fig. 2B and C). Although PRV-1 viral load in whole blood was higher than PRV-2 loads, indicating more efficient replication of PRV-1 in blood cells, comparing PRV-1 and PRV-2 induced gene expression did not reveal any significant expression differences after two weeks. This is consistent with the PCA plot, where data from PRV-1- and PRV-2- injected individuals clustered together (Supplementary File B, Fig. A1). A comparison between PRV-1 and PRV-3 revealed 148 host genes exhibiting higher expression in PRV-3-infected blood, as opposed to only 6 genes expressed higher in PRV-1-infected blood. This, together with the higher numbers of DEGs upregulated for the PRV-3 infected group versus controls, may indicate a stronger and faster response to PRV-3 than PRV-1. Transcriptional differences between PRV-1 and vaccinated controls were only few at week two. By week five, PRV-1 infected blood showed distinct transcriptional differences compared to the PRV-2 injected group and vaccinated controls, with approximately 1000 genes higher expressed and 500 genes lower expressed in PRV-1 infected blood (Fig. 2C). It is worth noting that no differentially expressed genes were detected when comparing vaccinated with PRV-2 infected fish at week two, and only a total of 24 genes differed at week five Fig. 2B). A comparison between PRV-3 and PRV-2 revealed that at week two, 449 genes had significantly higher expression in PRV-3- infected blood, whereas only 19 genes showed lower expression (higher expression in PRV-2- infected). By week five, 509 genes were identified with higher expression in PRV-3- infected blood, compared to 259 genes with higher expression for PRV-2 (Supplementary File F, Fig. A1). A similar relationship was found between PRV-3 and vaccinated controls at both week two and five (Fig. 2B), based on gene numbers.

3.4. Categorization of differentially expressed genes (DEGs) into functional groups and heatmaps for targeted differential expression analysis

DEGs in whole blood of infected A. salmon compared to uninfected controls at week two were categorized into functional groups using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology (GO) enrichment analyses for biological process (GO:BP) and molecular function (GO:MF) (Supplementary File C, Table A1-3). Transcriptional analysis of whole blood from vaccinated fish (compared to uninfected control) revealed a total of 13 DEGs (Fig. 2A). Thus, KEGG and GO enrichment analyses were not applicable. The categorization of the DEGs between PRV-3 vs PRV-2 and PRV3 vs InPRV-1 are provided in Supplementary File F (Figure A2-3). It is worth noting that several genes identified in A. salmon whole blood were not categorized into any functional group (uncategorized genes).

Transcriptional responses in whole blood of PRV-1- injected fish were compared to PRV-2, -3 and InPRV-1 at weeks two and five. Functional groups and heatmaps of each comparison with PRV-1 are provided separately in Figs. 3, 6 and 8.

3.4.1. Comparison of whole blood transcriptional responses to PRV-1 versus PRV-3 over time

Enrichment analysis for DEGs with higher expression in PRV-1-infected blood cells compared to PRV-3, revealed only three functional groups related to innate and adaptive immune responses (Fig. 3A) At two weeks, PRV-1 infection resulted in the induction of 31 genes encoding proteins with transcription regulatory activity, while only 9 genes involved in immune system processes (Supplementary File C, Table A1). In comparison, PRV-3 infection led to induction of 147 genes involved in immune system processes and 99 genes associated with responses to intracellular and/or external stimuli (Supplementary File C, Table A2). Both PRV-1 and PRV-3 infection suppressed genes involved in apoptosis, transmembrane transporter and transcription regulator activity. PRV-1 suppressed 12 genes involved in the MAPK signaling pathway (Supplementary File C, Table A3).

PRV-1 and PRV-3 replicated at a similar level in A. salmon blood, but diverged in the ability to cause HSMI pathology. The comparison of whole blood gene expression in response to PRV-1 versus PRV-3 revealed a total of 154 DEGs at week two and only 2 at week five (Fig. 2C). Focusing on week two, 6 genes exhibited higher expression level in response to PRV-1 and 148 genes in response to PRV-3 (Fig. 2C). Indicatively, genes such as proteasome 26 S subunit ATPase 3 (*PSMC3*)

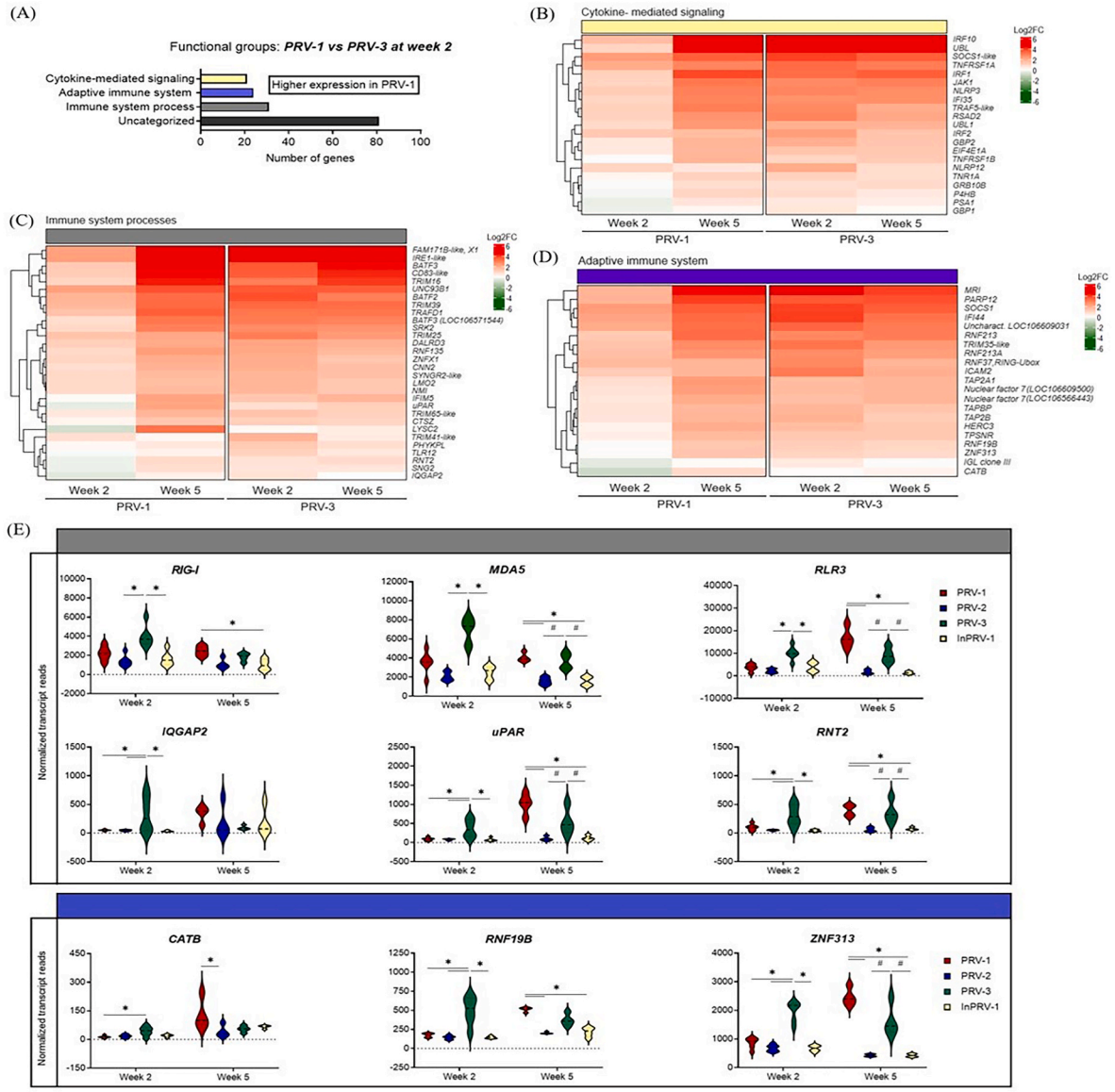

Fig. 3. Gene expression profile in whole blood of A. salmon injected with PRV-1 and PRV-3. DEGs with fold-change >2 (higher expression induced by PRV-1) and <0.5 (lower expression induced by PRV-1) were included in the analysis. Log2-fold change of the selected DEGs compared to uninfected controls at week zero. Red: Higher expression level at week two and/or five compared to week zero; Green: Lower expression level at week two/five compared to week zero; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A) Functional groups of DEGs between PRV-1 and PRV3 at week two. (B) Gene expression pattern of DEGs between PRV-1 and PRV-3, involved in immune system processes over time, compared to uninfected fish at week zero. (C) Gene expression pattern of DEGs between PRV-1 and PRV-3, involved in adaptive immune system, compared to uninfected fish at week zero. (D) Gene expression pattern of DEGs between PRV-1 and PRV-3, involved in cytokine mediated signaling, compared to uninfected fish at week zero. The colored band at the top of each heatmap corresponds to the functional group shown in A. (E) Expression levels of selected genes involved in "Immune system process" and "Adaptive immune system" groups as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes or vaccinated. *: $p \leq 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

interacting protein (*PSMC3IP*) involved in meiotic recombination, and cell surface protein tetraspanin 8-like (*TSPAN8*), showed higher expression levels in PRV-1 only. In contrast, genes with higher expression level in PRV-3 were primarily associated with innate and adaptive immune processes (Fig. 3D). The expression pattern of these genes in response to PRV-1 became equivalent to PRV-3 by week five. A few genes, such as IQ motif containing GTPase activating protein 2 (*IQGAP2*), urokinase plasminogen receptor (*uPAR*) and lysosomal protease cathepsin B (*CATB*), were slightly inhibited in response to PRV-1 at week two. However, their expression levels exceeded those induced by PRV-3 by week five (Fig. 3B–C, E).

Some uncategorized genes were strongly induced by PRV-3 at week two, including, interferon-induced protein with tetratricopeptide repeats 9 (IFIT9) and galectin 9 (LEG9) (Fig. 4). These genes are also involved in activation of innate immunity and antiviral defense and have previously been found induced in A. salmon erythrocytes in response to PRV-1 *in vivo* at later stages of infection [22]. Genes such as protein phosphatase Mg^{2+}/Mn^{2+} dependent (PPM) 1H (*PPM1H*) and *PPM1F*, as well as RNA binding motif protein 38 (*RBM38*), potentially associated with cell survival and viral genome replication [39,40], also exhibited high differential expression in PRV-3 infected relative to control blood at week two (Figs. 3 and 4, respectively). DEGs of all identified functional groups and uncategorized DEGs in PRV-1 and PRV-3 at week two are provided in Supplementary File E.

At week five, the two genes with higher expression induced by PRV-1 compared to PRV-3 encode barrier-to-autointegration factor (BANF) b

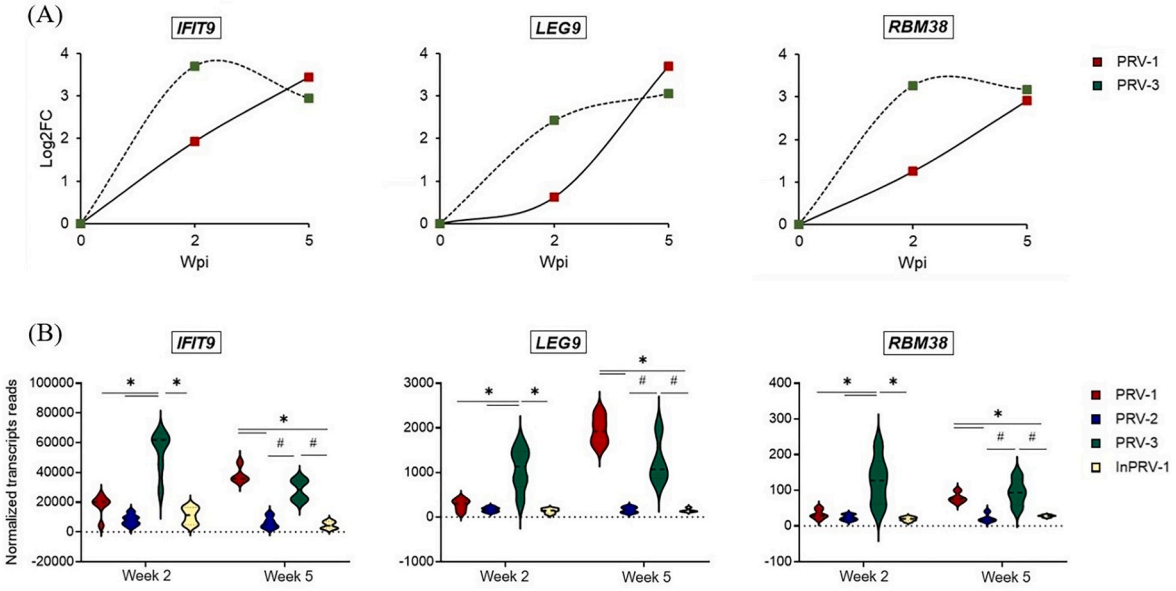

Fig. 4. Examples of uncategorized genes with higher relative expression in PRV-3 compared to PRV-1 infected blood at two and five weeks post infection. (A) DEGs with higher relative expression induced by PRV-3 than by PRV-1. Log2FC of DEGs between PRV1 and PRV-3 was calculated relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of the same genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes or vaccinated. *: $p \leq 0.01$.

and BANF-like DNA-binding protein (Fig. 5A). In mammals, these genes take part in various biological processes, such as transcription regulation, DNA damage response and innate immunity against viruses [41]. Infection with PRV-1 triggered the strongest transcriptional response of BANFB and BANF-like compared to other PRV genotypes and vaccinated controls (Fig. 5B).

3.4.2. Comparison of whole blood transcriptional responses to PRV-1 compared to PRV-2 over time

Whole blood from PRV-2 infected fish showed 88 genes with lower expression level compared to uninfected controls, but only 3 categories were generated from the GO and KEGG analysis. These genes belonged to functions related to MAPK signal transduction, protein folding and apoptosis (Supplementary File C, Table A3).

PRV-2 did not exhibit the same level of replication in whole blood of A. salmon compared to PRV-1 and PRV-3, but showed similar host gene expression profile to PRV-1 at week two. DEGs between PRV-1 and PRV-2 were explored to identify gene expression associated with virus replication. Only the phospholipase *DDHD1*-like (*DDHD1A*) gene exhibited 2-fold higher expression in PRV-2- relative to PRV-1- infected blood (raw data file- BioProject PRJNA1148351).

Although gene expression in whole blood of A. salmon in response to PRV-1 and PRV-2 was equivalent at week two, many genes were differentially expressed between the two genotypes by week five

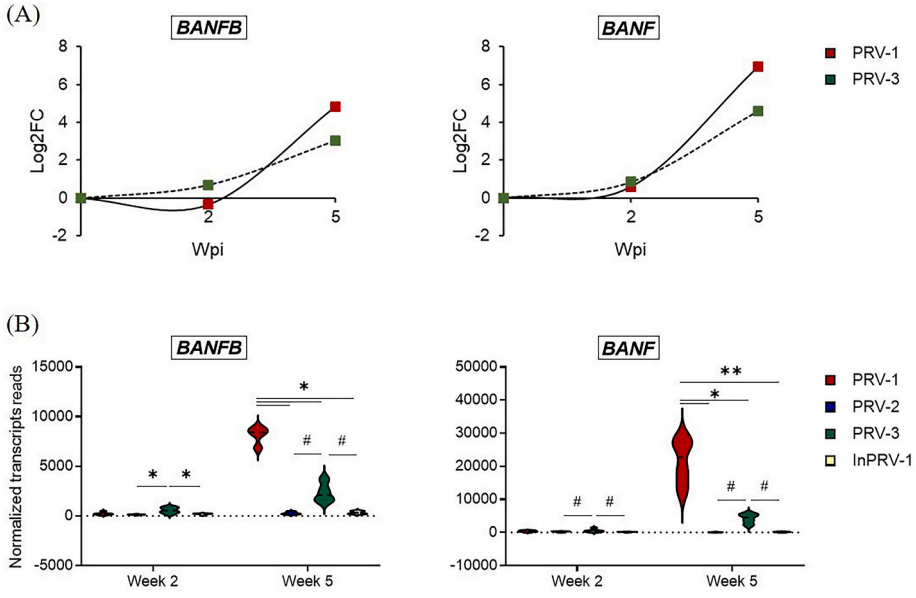

Fig. 5. Expression profile of barrier-to-autointegration factor (BANF) b and BANF-like DNA-binding protein genes overtime. BANFB and BANF were the only two genes with significantly higher expression in PRV-1 infected blood relative to PRV-3 at week five. (A) Log2FC of BANFB and BANF between PRV1 and PRV-3 relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of BANFB and BANF genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes and inactivated PRV-1 vaccine. *: $p \leq 0.01$.

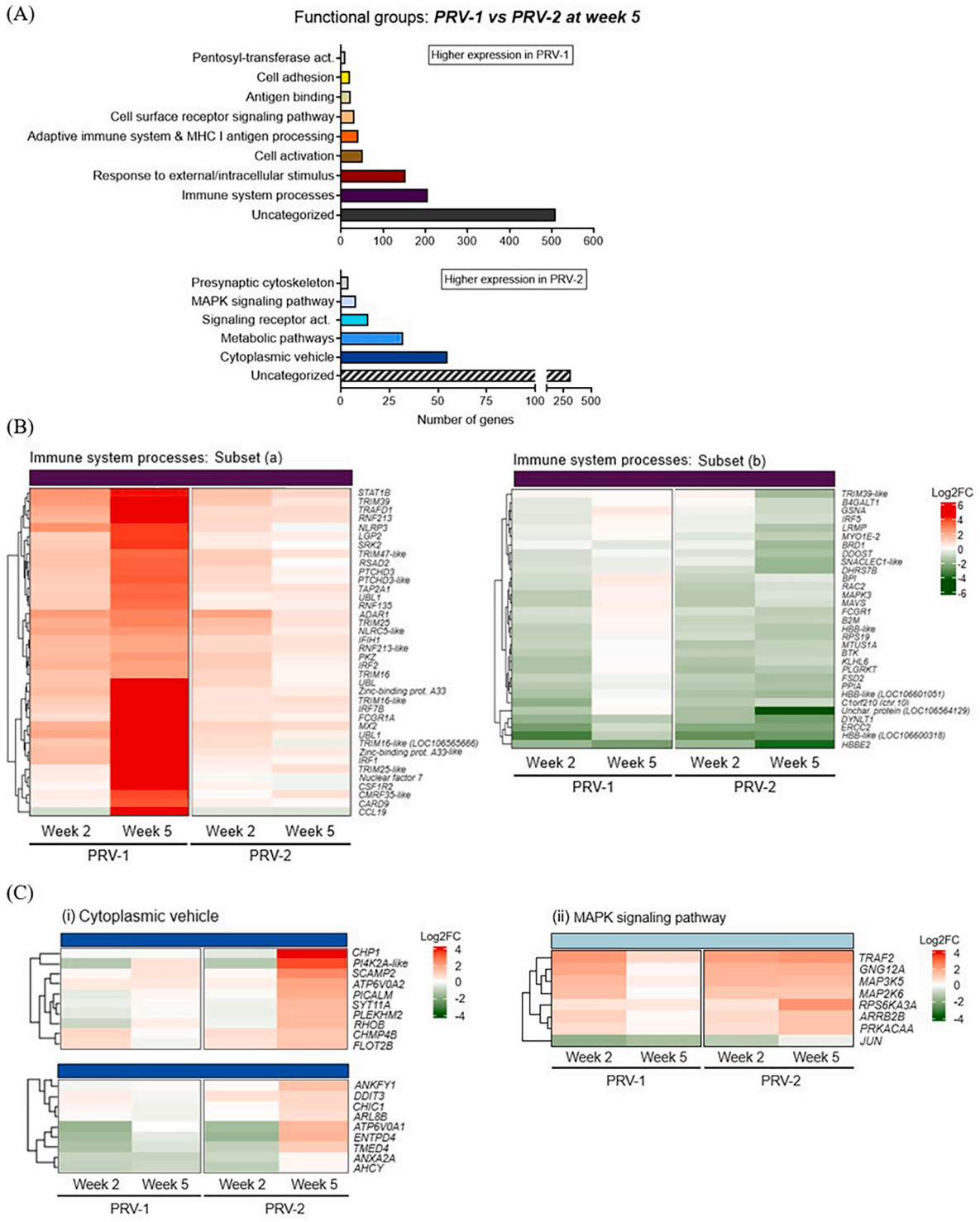

Fig. 6. Gene expression profile in whole blood of A. salmon injected with PRV-1 and PRV-2. DEGs with fold-change >2 (higher expression induced by PRV-1) and <0.5 (lower expression induced by PRV-1) were included in the analysis. Log2-fold change of the selected DEGs were compared to uninfected controls at week zero. Red: Higher expression level at week two and/or five; Green: Lower expression level at week two/five; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A) Functional groups of DEGs with higher and lower expression in PRV-1 (top and bottom, respectively) compared to PRV-2 at week five. (B) Transcriptional profile of DEGs between PRV-1 and PRV-2, involved in immune system processes (left) and identified functional groups (right) compared to uninfected fish at week zero. The colored panel to the left of each heatmap corresponds to a functional group from Fig. 6A (top). (C) Gene expression pattern of selected functional groups "(i) Cytoplasmic vehicle" and "(ii) MAPK signaling pathway". The colored panel at the top of each heatmap corresponds to a functional group from A (bottom). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 2C). Setting aside the group of uncategorized genes, the majority of DEGs with higher expression in PRV-1 was involved in immune system processes and signaling pathways activated in response to various stimuli (Fig. 6A, top). Transcriptional effectors typically involved in regulation of innate immune gene responses, such as signal transducer and activator of transcription 1 B (*STAT1B*) and TRAF-type zinc finger domain-containing protein 1 (*TRAFD1*) genes, together with genes related to antiviral defense, such as interferon regulatory factor (*IRF*) 1, *IRF7* and myxovirus resistance protein 2 (*MX2*), exhibited the highest expression levels in PRV-1- infected blood at week five. In comparison, no alterations in the expression profile of the same genes were observed in response to PRV-2 over time (Fig. 6B). Some genes associated with signal transduction and immune defense, including kinases (e.g. mitogen-activated protein kinase 3 (*MAPK3*)), small GTPases (e.g. Ras-related C3 botulinum toxin substrate 2 (*RAC2*)) and intermediary adapters (e.g. mitochondrial antiviral signaling protein (MAVS)), were expressed lower two weeks after both PRV-1 and PRV-2 exposure (Fig. 6B, subset b). However, by week five, they were slightly higher expressed in response to PRV-1, while showing even lower expression in response to PRV-2, compared to uninfected controls from week zero (Fig. 6B, subset b).

A total of 428 genes exhibited higher expression levels in response to PRV-2 at week five compared to PRV-1 (Fig. 2C). Gene ontology and KEGG pathway enrichment analyses revealed five main functional groups, as shown in the lower panel of Fig. 6A. The genes were implicated in intracellular trafficking, potentially associated with protein folding and degradation through vehicles, as well as in metabolic processes and signal transduction involving the activation of nuclear receptor subfamily members and MAP kinases.

Although many genes within the A. salmon genome were not grouped into specific cellular functions (Fig. 6A, bottom, "Uncategorized genes"), their counterparts in mammalian cells have been studied. For instance, genes encoding regulatory proteins, such as ring-finger protein 182 (*RNF182*) and dual specificity phosphate 11 (*DUSP11*), along with regulators of programmed cell death, such as calcium binding adaptor protein EF Hand domain family member D2 (*EFHD2*) were among genes with the highest differential expression in response to PRV-2 relative to control, in contrast to their significantly lower differential expression in response to PRV-1 at week five (Fig. 7). DEGs linked to specific functional groups and uncategorized DEGs induced by PRV-1 and PRV-2 at weeks two and five are provided in Supplementary File E.

3.4.3. Comparison of whole blood transcriptional responses to PRV-1 and InPRV-1 vaccine

The gene expression profile in whole blood of A. salmon infected by PRV-1 and vaccinated with InPRV-1 had only 58 DEGs at week two (Fig. 2B), despite the total inactivation and adjuvant added to the vaccine, and the high level of replication for PRV-1. By week five, the groups exhibited significant divergence, with 1583 genes differentially regulated (Fig. 2B). To elucidate the differences in transcriptional responses to InPRV-1 and PRV-1 over time, we first generated a heatmap for the 58 DEGs at week two (25 genes - higher expression induced by PRV-1; 28 genes - higher expression induced by InPRV-1) (Supplementary file D, Fig. A1). Genes involved in regulation of immune functions (e.g. *IRF1*, *TRAFD1*, *BATF3* and *IFI44*) and host genome replication (e.g. MCM2, -3, and -6) showed higher expression levels in the PRV-1 infected group at week two, which further increased by week five. This is consistent with PRV-1 being an actively infecting and replicating virus, distinguishing it from the inactive InPRV-1.

In general, most genes involved in innate and adaptive immunity exhibited a similar expression pattern in whole blood of A. salmon injected with PRV-1 - and InPRV-1 after two weeks. Only a few regulatory transcription factors involved in immune responses showed higher expression level in response to PRV-1 at the early infection stage. However, by week five, PRV-1 infection strongly induced genes related to antiviral defense, cytokine production and MHC I antigen processing, responses not further induced, and for some genes, even suppressed by InPRV-1. The responses are as expected due to increasing PRV-1 RNA levels in whole blood over time. Genes involved in diverse biological functions, such as responses to stimuli, cell-cell adhesion, pentosyltransferase activity and necrotic cell death, were also induced only in response to replicating PRV-1 (Supplementary File D, Fig. A1). Only a few genes with higher expression in InPRV-1 were identified with specific biological functions using GO and KEGG pathway enrichment analyses (Fig. 8A). Examples were genes encoding proteins located in lysosomes, and proteins that regulate intracellular transport through vacuoles (Fig. 8B). For instance, vacuolar protein sorting-associated proteins (*VPSs*) and ATPase H+ transporting V0 subunit A1 (*ATP6V0A1*) genes, were highly expressed only in response to InPRV-1 at week five. DEGs linked to specific functional groups and uncategorized DEGs for the PRV-1 and InPRV-1 comparison at weeks two and five is provided in Supplementary File E.

Interestingly, some genes related to MHC class I antigen processing and adaptive immunity, such as histocompatibility 2 Q region locus 10 (*H2-Q10*), MHC class I-related gene protein-like (*MR1*) and *TSPAN31*

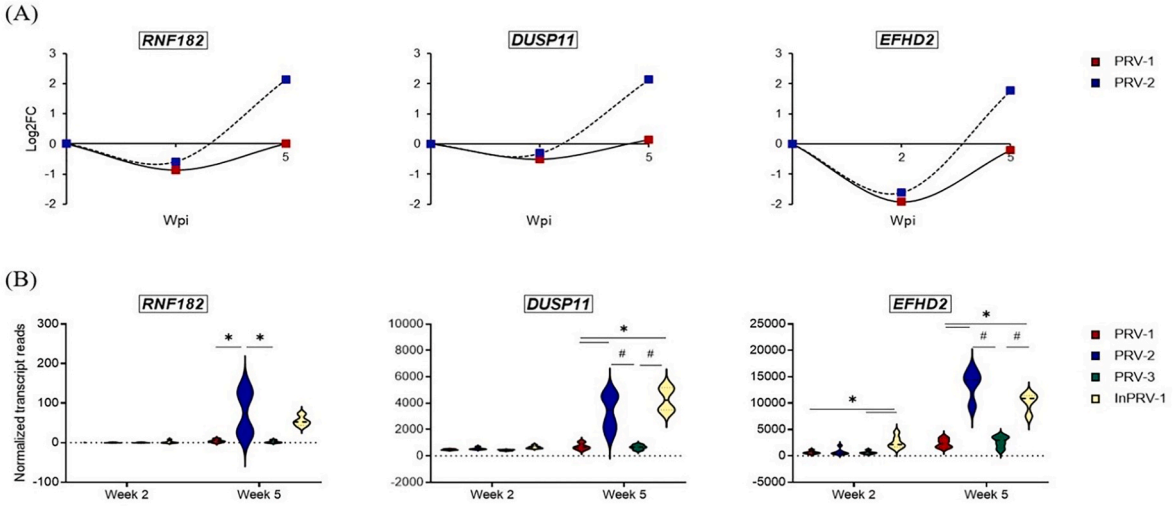

Fig. 7. Examples of uncategorized genes with higher relative expression in PRV-2 compared to PRV-1 infected blood at weeks two and five. (A) DEGs with higher relative expression induced by PRV-2. Log2FC of DEGs was calculated relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes and the inactivated PRV-1 vaccine. *: $p \leq 0.01$.

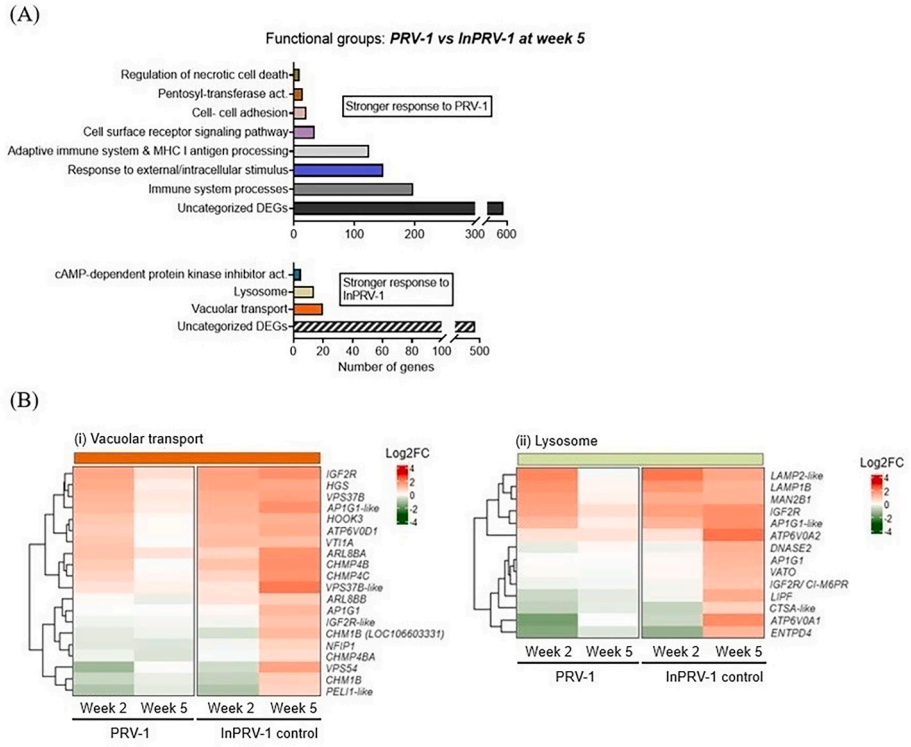

Fig. 8. Gene expression profile in whole blood of A. salmon injected with PRV-1 and InPRV-1 vaccine. DEGs with fold-change >2 (higher expression in PRV-1) and <0.5 fold-change (lower expression in PRV-1) were included in the analysis. Log2-fold change of the selected DEGs compared to uninfected controls at week zero. Red: Higher expression level at week two/five; Green: Lower expression level at week two/five; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A) Functional groups of DEGs with higher and lower expression in PRV-1 (top and bottom, respectively) compared to InPRV-1 at week five. (B) Transcriptional regulation of DEGs induced by PRV-1 compared to InPRV-1 within the identified functional groups (i) Vacuolar transport and (ii) Lysosome. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

showed higher transcription levels in the blood of vaccinated and PRV-2 infected fish compared to replicating PRV-1 and PRV-3 five weeks post infection. In particular, these genes exhibited >2-fold higher expression to InPRV-1 vaccine relative to uninfected controls at week five (Fig. 9). A complete overview of DEGs linked to specific functional groups and uncategorized DEGs in response to PRV-1 and vaccinated controls at weeks two and five is provided in Supplementary File E.

4. Discussion

The outcome of the infections with the three known PRV genotypes are different in A. salmon. They differ in the efficacy of replication in

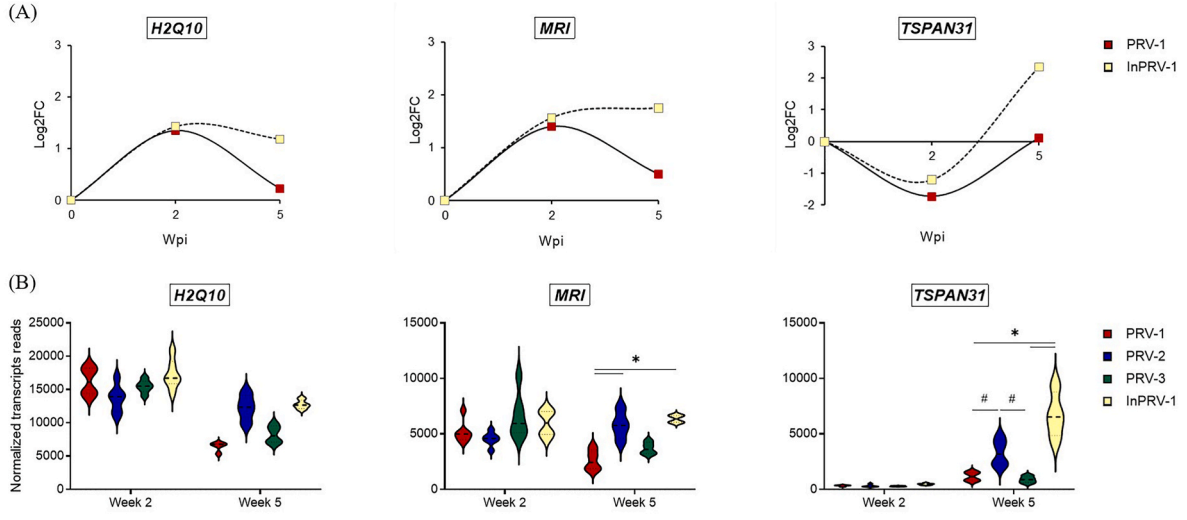

Fig. 9. Examples of genes with higher relative expression in vaccinated controls (InPRV-1) compared to PRV-1 at weeks two and five. (A) DEGs with higher relative expression in vaccinated controls compared to PRV-1 infected fish. Log2FC of DEGs for PRV1 and vaccinated controls was calculated relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes and the inactivated PRV-1 vaccine. *: $p \leq 0.01$.

blood (PRV-1 > PRV-3 > PRV-2), induction of heart pathology (PRV-1 only), and their potential to cross protect against secondary infection (PRV-3 > InPRV-1 vaccine > PRV-2) [29]. In this study, we explored whether gene expression differences in blood cells in response to the three PRV genotypes and an inactivated vaccine could provide more information on mechanisms of replication, pathogenesis and cross-protection.

4.1. Transcriptional responses in blood cells potentially linked to PRV replication

The three PRV genotypes showed relatively similar RNA loads in whole blood of A. salmon two weeks post injection of the virus, with somewhat higher levels of the pathogenic PRV-1 and the non-pathogenic PRV-3, compared to PRV-2 [29]. Three weeks later (week five) PRV-1 and PRV-3 continued to replicate in blood, PRV-1 to somewhat higher levels than PRV-3, whereas PRV-2 RNA levels were reduced in blood, indicating no further replication. Based on this replication pattern one could expect to see a similar cellular response to PRV-1 and PRV-3 and a divergent response to PRV-2. Interestingly, this was not the case after two weeks. While PRV-3 triggered a strong transcriptional response after two weeks relative to uninfected and vaccinated controls, blood cells showed a much lower response to both PRV-1 and PRV-2. After five weeks, PRV-1 had replicated to the highest level in blood, and as expected regulated the highest number of host genes relative to vaccinated controls. PRV-3 had also replicated to higher levels between two and five weeks, and the transcriptional responses to the virus remained relatively similar to responses at week two. Responses to PRV-2 were still low, in line with the lack of further replication.

PRV-3 induced the strongest innate antiviral responses at week two, with the transcriptional levels of most genes remaining relatively stable until week five, while the virus continued to replicate. This indicates that the innate immune gene expression was initiated long before the virus reached the highest replication levels in blood cells, contradictory to responses to PRV-1, that peaked later [9,22]. Both the PRV-3 response at week two and five, and the strong response to PRV-1 at week five were characterized by effects on a similar set of innate antiviral genes, indicating that the difference in response was mainly associated with timing. Genes associated with a typical dsRNA-induced antiviral response, including PRRs (e.g. *RLR1, RLR3* and *MDA5)*, transcription regulators (e.g. *IRF-3/7)*, cytokine signaling mediators (e.g. *JAK1*, *STAT1*, galectin 9 (*LEG9*)) and IFN- inducible effectors (e.g. *Mx2* and *ISG15)*, have previously been associated with progression of PRV-1 infection in RBCs *in vivo* [2,22], studied mainly in the period between the viral peak in blood and the onset of HSMI, and not during early infection [15,22,29]. PRV-1 infection is persistent in blood cells, and previous research has indicated that the antiviral response to the virus is long lasting [29]. PRV-3 RNA is also shown to persist in infected A. salmon for at least 10 weeks, but the antiviral responses appear to weaken over time [29].

Given the genetic variation in PRV sequence identity, modest responses to PRV-2 may be attributed to a low rate of amino acid (aa) conservation in segments essential for viral binding, entry and/or replication, compared to host-specific PRV-1 [42]. In this sense, PRV-2 may have not been internalized and the dsRNA genome not sensed by A. salmon RBCs, in line with low replication potential. This is further supported when investigating the expression of genes related to viral dsRNA recognition, such as *TLR3*, *RLR1*, *RLR3* and *MDA5*, and transcription regulation, such as *IRF-1* and IRF-3/7 that did not respond to PRV-2. Interestingly, five weeks post PRV-2 exposure, the genes encoding ring finger protein 182 (*RNF182*) and dual-specificity phosphatase 11 (*DUSP11*) were significantly induced only in response to PRV-2. In mammals, *RNF182* and *DUSP11* interact with *TLR3* and *RLR1*, respectively, suppressing the IFN- mediated pathway and antiviral defense [43,44]. This may also explain the limited activation of innate immune antiviral responses to PRV-2 over time. An interesting observation in this context, is that the fish injected with PRV-2, when exposed to a secondary PRV-1 infection, PRV-1 appeared to replicate more efficiently in spleen of some individuals, reaching maximal Ct levels of 10–12, compared to peak Ct levels of 13–14 in PRV-1 control groups [29]. Therefore, the activation of such genes by PRV-2, with inhibitory effect in viral genome recognition and initiation of antiviral responses, may also favor PRV-1 to replicate more efficiently upon consecutive infection.

PRV-3 exhibits higher genetic similarity to PRV-1 [42] in genomic segments which were previously suggested to facilitate virus internalization, propagation and persistence in blood cells and spleen of A. salmon [29]. However, the mechanisms of PRV-3 interaction with A. salmon RBCs may be less evolutionary adapted. Similar to MRV infection mechanism, PRV-1 and PRV-3 likely enters A. salmon RBCs through receptor-dependent endocytosis [45]. The outer part of the double capsid of the internalized virion is partly disassembled in the endosomes and the inner capsid containing the dsRNA genome is transferred to the cell cytoplasm. There, viral factories are formed to serve as production sites for viral amplification, keeping the dsRNA genome protected from exposure to host immune defense mechanisms [25]. While PRV-3 appeared to replicate equally well as PRV-1 in A. salmon RBCs, differences in the infection mechanisms may have led to more exposure of the dsRNA genome in PRV-3 infected cells, a putative explanation on why antiviral responses were triggered earlier.

4.2. Are specific transcriptional responses to PRV-1 linked to dissemination and HSMI?

Although PRV-3 may infect and replicate in A. salmon blood cells, and elicit strong antiviral responses when injected IP, transmission of PRV-3 does not occur naturally from infected to naïve cohabitant A. salmon [29,46]. For PRV-1, transmission to shedders is associated with the early phase of replication when intact virus is released from RBCs to blood plasma. This phase is also associated with virus dissemination to the heart, preceding HSMI [28]. PRV is shown to shed through feces, and also to infect new individuals over the intestinal mucosa [47]. It is unknown whether the lack of transmission of PRV-3 in A. salmon is due to the virus low ability to disseminate into blood plasma and/or be shed through feces, or if PRV-3 is shed but unable to cross mucosal surfaces and enter the blood stream. Previous observations indicate that PRV-3 does not infect the A salmon heart to the same degree as PRV-1, which may explain why PRV-3 does not lead to HSMI [29,46]. This points towards the dissemination step into blood plasma as the determining factor and indicates that replication and antiviral mechanisms in RBCs may be key. Thus, PRV-1 may escape host antiviral responses and promote its replication/dissemination through inhibition and delay of the same antiviral mechanisms that inhibit PRV-3 dissemination.

For instance, the genes encoding *IQGAP2* and urokinase plasminogen activator receptor (*uPAR*) protein showed lower expression two weeks post PRV-1 infection relative to uninfected controls. In contrast, their expression in response to PRV-3 was significantly higher. The mammalian *IQGAP2* gene serves as an IFN effector, essential for the transcription activation of IFN stimulated genes in response to viral encounter, through interaction with the P65 subunit of nuclear factor-κB (NF- κB) complex [48]. While NF- κB has been characterized as a critical component in reovirus replication and apoptosis induction in host cells in general, so far the involvement in PRV infection of A. salmon RBCs is not evident [2,22,23]. Neither NF- κB nor subunits P50 and P65 were induced at the transcriptional level in response to any PRV genotypes here, but may be activated at a post-transcriptional level. Although the *IQGAP2* regulation may have a critical role in host-virus interaction in teleosts, similar to NF- κB in MRV [48], this mechanism is yet to be explored. The protein *uPAR* (also known as CD87) serves a role in immune cell adhesion and migration [49], and is upregulated in response to many viruses [50]. In mammals, it is also implicated in blockage of HIV release from the infected blood cells at the late phase of viral replication [51]. In this sense, inhibition of *uPAR* transcription by PRV-1

at early stages of infection may favor its release into/from RBCs before the initiation of innate immune responses. It is worth noting that a recent proteomic analysis concluded *uPAR* protein as a good candidate biomarker for PRV-1 pathogenesis due to its elevated levels in plasma of PRV-1 positive A. salmon, primarily in association with HSMI onset [52]. The *uPAR* gene activation in blood as reported in our study at week two and five, may further strengthen its biomarker potential.

Another set of genes that showed distinctively high response only to PRV-1 at both two and five weeks post infection were the barrier to autointegration factor proteins *BANFB* and *BANF*. These genes are implicated in host protection by intercepting foreign genomes, a mechanism previously shown to be exploited by mammalian retroviruses to enhance their replication and prevalence in host cells [41]. In addition, *BANF*, in association with serine-threonine vaccinia-related kinases (VRKs) 1 and 3, whose high expression was also induced primarily by PRV-1 infection, is involved in maintaining the integrity of nuclear envelope. Acting as a transcription regulator, *BANF* is also implicated in signaling pathways, potentially moderating gene expression and cell survival [53]. While the functionality of *BANFB* and *BANF* in fish is poorly understood, their overexpression in whole blood of PRV-1 infected fish may suggest their implication in cellular functions associated with both viral replication and persistence mechanism, and the observed survival of infected RBCs [16]. In addition, given the significantly high transcriptional levels of *BANFB* and *BANF* only in response to PRV-1, future investigation of these genes and their proteins as potential biomarkers of PRV-1 infection and pathogenicity may be worth exploring.

MRV infection has been reported to induce the release of lysosomal *CATB* protein, which serves as a "danger" signal activating host innate immune responses [54,55]. According to this, inhibition of *CATB* expression, observed only in PRV-1 infected blood cells, may also constitute a viral mechanism of immune evasion, not evolved for PRV-3. Other genes encoding lysosomal proteins, such as ribonuclease T2 (*RNT2* or *RNASET2*), exhibited similar expression profile to *CATB* in PRV-1 and PRV-3 infected blood cells, which may also suggest their implication in virus-host interaction mechanisms.

4.3. Transcriptional changes induced by non-replicating PRV genotypes

Vaccination of A. salmon with adjuvanted InPRV-1 has been shown to induce moderate protection against consecutive PRV-1 infection, primarily when fish were challenged through cohabitation [29,30]. Here, the transcriptional profile of genes implicated in mediation of innate and adaptive immunity was similar in whole blood of vaccinated and PRV-2 infected fish, both at week two and five. However, InPRV-1 vaccine blocked PRV-1 infection and HSMI more efficiently than PRV-2 [29]. This may indicate that physiological and immunological events (e.g. T-cell responses) taken place in other lymphoid tissues primarily contributed to this outcome. It is worth noting that the adjuvant used in the preparation of InPRV-1 vaccine has not been revealed in detail, and the interpretation of the data should consider that the observed responses may both result from the adjuvant, or from the inactivated virus itself. No PRV-1 specific antibodies have been demonstrated after InPRV-1 vaccination [29].

Genes implicated in adaptive immunity and major histocompatibility complex (MHC) class I antigen processing in blood cells were significantly induced primarily by PRV-3, in a manner similar to PRV-1, which may coincide with the efficient protection of A. salmon against PRV-1 and HSMI. Interestingly, some genes involved in MHC class I antigen presentation in mammals, such as histocompatibility 2 Q region locus 10 (*H2-Q10*) and MHC class I-related gene protein-like (*MR1*) [56,57], exhibited significantly higher expression in blood cells of vaccinated and PRV-2 infected fish relative to PRV-1 and PRV-3. The same was also observed for the genes encoding EF-hand domain-containing protein D2 (*EFHD2*), associated with regulation of T cell-mediated inflammation [58] and *TSPAN31*, linked to apoptosis through a PI3K/Akt pathway [59]. Although the observed transcriptional differences elicited by the PRV genotypes in whole blood may be linked to differential pathogenicity and cross-protection in A. salmon, the functional role of these genes in salmonid blood cells needs to be further explored.

5. Conclusion and future perspectives

This transcriptional study demonstrated that PRV-3, a cross-protective PRV-genotype that does not cause HSMI in A. salmon, triggered potent innate antiviral responses during early replication in blood cells. In comparison, PRV-1 triggered a delayed but similar antiviral response. This difference in the timing of antiviral response may provide an evolutionary advantage for PRV-1, allowing it to disseminate and infect the A salmon heart, a prerequisite for HSMI onset. The early and robust antiviral response to PRV-3 in blood cells likely contributes to the subsequent production of cross-binding *anti*-PRV antibodies, efficiently blocking PRV-1 infection and the development of HSMI.

Currently, the mechanisms of PRV entry-exit and dissemination within the host cells remain poorly understood. Functional studies on interesting gene candidates with differential expression between PRV-1 and PRV-3, such as *IQGAP2* and *BANF*, may help establish their role in RBCs and viral transmission to cardiomyocytes. Addressing these knowledge gaps would significantly enhance the understanding of the mechanisms involved in PRV pathogenesis and cross-protection.

CRediT authorship contribution statement

Thomais Tsoulia: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Arvind YM. Sundaram:** Data curation, Formal analysis, Methodology, Supervision, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Marit M. Amundsen:** Formal analysis, Methodology, Validation, Writing – review & editing. **Espen Rimstad:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Øystein Wessel:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Jorunn B. Jørgensen:** Investigation, Supervision, Validation, Writing – review & editing. **Maria K. Dahle:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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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.2024.110088.

Data availability

The data can be found here: NCBI SRA BioProject- PRJNA1148351 Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of Piscine orthoreovirus (Original data) (NCBI SRA)

References

[1] D. Morera, S.A. MacKenzie, Is there a direct role for erythrocytes in the immune response? Vet. Res. 42 (1) (2011) 1–8, https://doi.org/10.1186/1297-9716-42-89.

[2] D. Morera, N. Roher, L. Ribas, J.C. Balasch, C. Doñate, A. Callol, S. Boltana, S. Roberts, G. Goetz, F.W. Goetz, S.A. MacKenzie, Rna-seq reveals an integrated immune response in nucleated erythrocytes, PLoS One 6 (10) (2011), https://doi.org/10.1371/JOURNAL.PONE.0026998.

[3] T. Markussen, M.K. Dahle, T. Tengs, M. Løvoll, Ø.W. Finstad, C.R. Wiik-Nielsen, S. Grove, S. Lauksund, B. Robertsen, E. Rimstad, Sequence analysis of the genome of piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in atlantic salmon (Salmo salar), PLoS One 8 (7) (2013), https://doi.org/10.1371/journal.pone.0070075.

[4] G. Palacios, M. Lovoll, T. Tengs, M. Hornig, S. Hutchison, J. Hui, R.T. Kongtorp, N. Savji, A.V. Bussetti, A. Solovyov, A.B. Kristoffersen, Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus, PLoS One 5 (7) (2010) 3–9, https://doi.org/10.1371/journal.pone.0011487.

[5] Ø.W. Finstad, M.K. Dahle, T.H. Lindholm, I.B. Nyman, M. Løvoll, C. Wallace, C. M. Olsen, A.K. Storset, E. Rimstad, Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes, Vet. Res. 45 (1) (2014) 1–13, https://doi.org/10.1186/1297-9716-45-35.

[6] Ø. Wessel, C.M. Olsen, E. Rimstad, M.K. Dahle, "Piscine Orthoreovirus (PRV) Replicates in Atlantic Salmon (Salmo salar L.) Erythrocytes Ex Vivo, 2015, https://doi.org/10.1186/s13567-015-0154-7.

[7] I. Sommerset, J. Wiik-Nielsen, T. Modal, V.H.S. Oliveira, J.C. Svendsen, A. Haukaas, E. Brun, Norwegian fish health report 2022, Publ. by Nor. Vet. Inst. (2023) series #5a/2023, 2023, https://www.barentswatch.no/en/fishhealth/.

[8] G. Polinski, N. Vendramin, A. Cuenca, K.A. Garver, Piscine orthoreovirus: biology and distribution in farmed and wild fish, J. Fish. Dis. 43 (11) (2020) 1331–1352, https://doi.org/10.1111/jfd.13228.

[9] Ø. Wessel, S. Braaen, M. Alarcon, H. Haatveit, N. Roos, T. Markussen, T. Tengs, M. K. Dahle, E. Rimstad, Infection with purified Piscine orthoreovirus demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon, PLoS One 12 (8) (2017) e0183781, https://doi.org/10.1371/journal.pone.0183781.

[10] H.W. Ferguson, R.T. Kongtorp, T. Taksdal, D. Graham, K. Falk, An outbreak of disease resembling heart and skeletal muscle inflammation in Scottish farmed salmon, Salmo salar L., with observations on myocardial regeneration, J. Fish. Dis. 28 (2) (2005) 119–123, https://doi.org/10.1111/j.1365-2761.2004.00602.x.

[11] S. Soares, V. Romano, S. Ives, CMS, HSMI & PD Scottish Data 2022 & 2023, 2024.

[12] E. Biering, Å.H. Garseth, Heart and skeletal muscle inflammation (HSMI) of farmed Atlantic salmon (Salmo salar L.) and the associated Piscine reovirus (PRV), ICES Identif. Leafl. Dis. Parasites Fish Shellfish 58 (6) (2012). Available: www.ices.dk.

[13] M. Løvoll, M. Alarcón, B. Bang Jensen, T. Taksdal, A.B. Kristoffersen, T. Tengs, Quantification of piscine reovirus (PRV) at different stages of Atlantic salmon Salmo salar production, Dis. Aquat. Org. 99 (1) (2012) 7–12, https://doi.org/10.3354/dao02451.

[14] N.A. Vatne, M. Stormoen, M. Lund, M. Devold, E. Rimstad, Ø. Wessel, Genetic grouping and geographic distribution of Piscine orthoreovirus-1 (PRV-1) in farmed Atlantic salmon in Norway, Vet. Res. 52 (1) (2021) 131, https://doi.org/10.1186/s13567-021-01000-1.

[15] Ø. Wessel, E.F. Hansen, M.K. Dahle, M. Alarcon, N.A. Vatne, I.B. Nyman, K. B. Soleim, D. Kannimuthu, G. Timmerhaus, T. Markussen, M. Lund, H. Aanes, M. Devold, M. Inami, M. Løvoll, E. Rimstad, Piscine orthoreovirus-1 isolates differ in their ability to induce heart and skeletal muscle inflammation in atlantic salmon (Salmo salar), Pathogens 9 (12) (2020) 1–22, https://doi.org/10.3390/pathogens9121050.

[16] A.B. Olsen, M. Hjortaas, T. Tengs, H. Hellberg, R. Johansen, First description of a new disease in rainbow trout (Oncorhynchus mykiss (Walbaum)) similar to heart and skeletal muscle inflammation (HSMI) and detection of a gene sequence related to piscine orthoreovirus (PRV), PLoS One 10 (7) (2015) 1–14, https://doi.org/10.1371/journal.pone.0131638.

[17] N. Vendramin, D. Kannimuthu, A.B. Olsen, A. Cuenca, L.H. Teige, Ø. Wessel, T. M. Iburg, M.K. Dahle, E. Rimstad, N.J. Olesen, Piscine orthoreovirus subtype 3 (PRV-3) causes heart inflammation in rainbow trout (Oncorhynchus mykiss), Vet. Res. 50 (1) (2019) 1–13, https://doi.org/10.1186/s13567-019-0632-4.

[18] E. Di Cicco, H.W. Ferguson, K.H. Kaukinen, A.D. Schulze, S. Li, A. Tabata, O. P. Günther, G. Mordecai, C.A. Suttle, K.M. Miller, The same strain of Piscine orthoreovirus (PRV-1) is involved in the development of different, but related, diseases in Atlantic and Pacific Salmon in British Columbia, Facets 3 (1) (2018) 599–641, https://doi.org/10.1139/facets-2018-0008.

[19] J. Cartagena, C. Jiménez, E. Spencer, Detection of Piscine orthoreoviruses (PRV-1b AND PRV-3a) in farmed Coho salmon with jaundice syndrome from Chile, Aquaculture 528 (April) (2020) 735480, https://doi.org/10.1016/j.aquaculture.2020.735480.

[20] T. Takano, A. Nawata, T. Sakai, T. Matsuyama, T. Ito, J. Kurita, S. Terashima, M. Yasuike, Y. Nakamura, A. Fujiwara, A. Kumagai, Full-genome sequencing and confirmation of the causative agent of erythrocytic inclusion body syndrome in coho salmon identifies a new type of piscine orthoreovirus, PLoS One 11 (10) (2016) 1–20, https://doi.org/10.1371/journal.pone.0165424.

[21] C.D. Eckstrand, B.K. Torrevillas, R.M. Wolking, D.S. Bradway, J.L. Reno, K. M. McMenamin-Snekvik, K.R. Snekvik, Detection, sequencing, and tissue distribution of piscine orthoreovirus 2–like virus in diseased coho salmon in Alaska, J. Vet. Diagn. Invest. 36 (3) (2024) 338–345, https://doi.org/10.1177/10406387241250119. Available.

[22] M.K. Dahle, Ø. Wessel, G. Timmerhaus, I.B. Nyman, S.M. Jørgensen, E. Rimstad, A. Krasnov, Transcriptome analyses of Atlantic salmon (Salmo salar L.) erythrocytes infected with piscine orthoreovirus (PRV), Fish Shellfish Immunol. 45 (2) (Aug. 2015) 780–790, https://doi.org/10.1016/j.fsi.2015.05.049.

[23] T. Tsoulia, A.Y. Sundaram, S. Braaen, J.B. Jørgensen, E. Rimstad, Ø. Wessel, M. K. Dahle, Transcriptomics of early responses to purified Piscine orthoreovirus-1 in Atlantic salmon (Salmo salar L.) red blood cells compared to non-susceptible cell lines, Front. Immunol. 15 (February) (2024) 1–16, https://doi.org/10.3389/fimmu.2024.1359552.

[24] H.M. Haatveit, Ø. Wessel, T. Markussen, M. Lund, B. Thiede, I.B. Nyman, S. Braaen, M.K. Dahle, E. Rimstad, "Viral protein kinetics of piscine orthoreovirus infection in atlantic salmon, Blood Cell 9 (3) (2017) 49, https://doi.org/10.3390/v9030049.

[25] E.I. Zuniga, M. Macal, G.M. Lewis, J.A. Harker, Innate and adaptive immune regulation during chronic viral infections, Annu. Rev. Virol. 2 (2015) 573–597, https://doi.org/10.1146/annurev-virology-100114-055226.

[26] L.H. Teige, M. Lund, H.M. Haatveit, M.V. Røsæg, Ø. Wessel, M.K. Dahle, A. K. Storset, A bead based multiplex immunoassay detects Piscine orthoreovirus specific antibodies in Atlantic salmon (Salmo salar), Fish Shellfish Immunol. 63 (2017) 491–499, https://doi.org/10.1016/j.fsi.2017.02.043.

[27] L.H. Teige, S. Kumar, G.M. Johansen, Ø. Wessel, N. Vendramin, M. Lund, E. Rimstad, P. Boysen, M.K. Dahle, Detection of salmonid IgM specific to the piscine orthoreovirus outer capsid spike protein sigma 1 using lipid-modified antigens in a bead-based antibody detection assay, Front. Immunol. 10 (September) (2019) 1–12, https://doi.org/10.3389/fimmu.2019.02119.

[28] D. Kannimuthu, H. Bjørgen, M.S. Malik, I.B. Nyman, T.A. Markussen, M.K. Dahle, E.O. Koppang, Ø. Wessel, E. Rimstad, Dissemination of piscine orthoreovirus-1 (PRV-1) in atlantic salmon (Salmo salar) during the early and regenerating phases of infection, Pathog. MDPI (2020), https://doi.org/10.3390/pathogens9020143.

[29] M.S. Malik, L.H. Teige Lh, S. Braaen, A.B. Olsen, M. Nordberg, M.M. Amundsen, D. Kannimuthu, S. Svenning, E.S. Edholm, T. Takano, J.B. Jørgensen, Ø. Wessel, E. Rimstad, M.K. Dahle, Piscine orthoreovirus (PRV)-3, but not PRV-2, cross-protects against PRV-1 and heart and skeletal muscle inflammation in atlantic salmon, Vaccines 9 (3) (2021) 1–20, https://doi.org/10.3390/vaccines9030230.

[30] Ø. Wessel, Ø. Haugland, M. Rode, B.N. Fredriksen, M.K. Dahle, E. Rimstad, Inactivated Piscine orthoreovirus vaccine protects against heart and skeletal muscle inflammation in Atlantic salmon, J. Fish. Dis. 41 (9) (2018) 1411–1419, https://doi.org/10.1111/jfd.12835.

[31] H.M. Haatveit, K. Hodneland, S. Braaen, E.F. Hansen, I.B. Nyman, M.K. Dahle, P. Frost, E. Rimstad, 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), Vaccine 36 (50) (2018) 7599–7608, https://doi.org/10.1016/j.vaccine.2018.10.094.

[32] B. Bushnell, "BBTools Software package.", 2014.

[33] D. Kim, J.M. Paggi, C. Park, C. Bennett, S.L. Salzberg, Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype, Nat. Biotechnol. 37 (8) (2019) 907–915, https://doi.org/10.1038/s41587-019-0201-4.

[34] Y. Liao, G.K. Smyth, W. Shi, FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features, Bioinformatics 30 (7) (2014) 923–930, https://doi.org/10.1093/bioinformatics/btt656.

[35] H. Varet, L. Brillet-Guéguen, J.Y. Coppée, M.A. Dillies, SARTools: a DESeq2- and edgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data, PLoS One 11 (6) (2016) 1–8, https://doi.org/10.1371/journal.pone.0157022.

[36] R. D. C. Team, R: A Language and Environment for Statistical Computing, 2010.

[37] M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, Genome Biol. 15 (12) (2014) 1–21, https://doi.org/10.1186/s13059-014-0550-8.

[38] D. Szklarczyk, R. Kirsch, M. Koutrouli, K. Nastou, F. Mehryary, R. Hachilif, A. L. Gable, T. Fang, N.T. Doncheva, S. Pyysalo, P. Bork, The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest, Nucleic Acids Res. 51 (1 D) (2023) D638–D646, https://doi.org/10.1093/nar/gkac1000.

[39] A. Ishida, N. Sueyoshi, I. Kameshita, Functions and dysfunctions of Ca2+/calmodulin-dependent protein kinase phosphatase (CaMKP/PPM1F) and CaMKP-N/PPM1E, Arch. Biochem. Biophys. 640 (January) (2018) 83–92, https://doi.org/10.1016/j.abb.2018.01.001.

[40] S.S. Ganaie, A.Y. Chen, C. Huang, P. Xu, S. Kleiboeker, A. Du, J. Qiu, RNA binding protein RBM38 regulates expression of the 11-kilodalton protein of parvovirus B19, which facilitates viral DNA replication, J. Virol. 92 (8) (2018) 10–1128, https://doi.org/10.1128/jvi.02050-17.

[41] A. Jamin, M.S. Wiebe, Barrier to Autointegration Factor (BANF1): interwoven roles in nuclear structure, genome integrity, innate immunity, stress responses and progeria, Curr. Opin. Cell Biol. 34 (2015) 61–68, https://doi.org/10.1016/j.ceb.2015.05.006.

[42] D. Kannimuthu, N. Vendramin, T. Markussen, Ø. Wessel, A. Cuenca, I.B. Nyman, A. B. Olsen, T. Tengs, M. Dahle, E. Rimstad, Molecular and antigenic characterization

of piscine orthoreovirus (PRV) from rainbow trout (oncorhynchus orthoreovirumykiss), Viruses 10 (4) (2018) 1–16, https://doi.org/10.3390/v10040170.
[43] J.H. Choi, C.S. Sullivan, DUSP11 and triphosphate RNA balance during virus infection, PLoS Pathog. 17 (1) (2021) 1–7, https://doi.org/10.1371/journal.ppat.1009145.
[44] C. Cai, Y.D. Tang, J. Zhai, C. Zheng, The RING finger protein family in health and disease, Signal Transduct. Targeted Ther. 7 (1) (2022), https://doi.org/10.1038/s41392-022-01152-2.
[45] A.N. Roth, P. Aravamudhan, I. Fernández de Castro, R. Tenorio, C. Risco, T. S. Dermody, Ins and outs of reovirus: vesicular trafficking in viral entry and egress, Trends Microbiol. 29 (4) (2021) 363–375, https://doi.org/10.1016/j.tim.2020.09.004.
[46] H. Hauge, N. Vendramin, T. Taksdal, A.B. Olsen, Ø. Wessel, S.S. Mikkelsen, A. L. Alencar, N.J. Olesen, M.K. Dahle, "Infection Experiments with Novel Piscine Orthoreovirus from Rainbow Trout (Oncorhynchus mykiss) in Salmonids,", 2017, https://doi.org/10.1371/journal.pone.0180293.
[47] H. Hauge, M. Dahle, T. Moldal, E. Thoen, A.G. Gjevre, S. Weli, M. Alarcón, S. Grove, Piscine orthoreovirus can infect and shed through the intestine in experimentally challenged Atlantic salmon (Salmo salar L.), Vet. Res. 47 (1) (2016) 1–12, https://doi.org/10.1186/s13567-016-0343-z.
[48] C. Brisac, S. Salloum, V. Yang, E.A. Schaefer, J.A. Holmes, S. Chevaliez, J. Hong, C. Carlton-Smith, N. Alatrakchi, A. Kruger, W. Lin, IQGAP2 is a novel interferon-alpha antiviral effector gene acting non-conventionally through the NF-κB pathway, J. Hepatol. 65 (5) (2016) 972–979, https://doi.org/10.1016/j.jhep.2016.06.028.
[49] D. D'Alonzo, M. De Fenza, V. Pavone, COVID-19 and pneumonia: a role for the uPA/uPAR system, Drug Discov. Today 25 (8) (2020) 1528–1534, https://doi.org/10.1016/j.drudis.2020.06.013.
[50] J. Yu, V. Murthy, S.L. Liu, Relating GPI-Anchored Ly6 Proteins uPAR and CD59 to viral infection, Viruses 11 (11) (2019) 14–16, https://doi.org/10.3390/v11111060.
[51] H. Pang, J. Ouyang, Z. Yang, H. Shang, G. Liang, Urokinase plasminogen activator surface receptor restricts HIV-1 replication by blocking virion release from the cell membrane, Proc. Natl. Acad. Sci. USA 120 (3) (2017) 2017, https://doi.org/10.1073/pnas.
[52] C.K. Fæste, O.D. Rangel-Huerta, J.H. Anonsen, H. Tartor, R.V. Kuiper, M.K. Dahle, Potential plasma biomarkers for the onset of heart and skeletal muscle inflammation from Piscine orthoreovirus-1 infection in Atlantic salmon (Salmo salar), Aquaculture 590 (December 2023) (2024) 741045, https://doi.org/10.1016/j.aquaculture.2024.741045.
[53] R.M. Sears, K.J. Roux, Diverse cellular functions of barrier-to-autointegration factor and its roles in disease, J. Cell Sci. 133 (16) (2020), https://doi.org/10.1242/jcs.246546.
[54] D.H. Ebert, J. Deussing, C. Peters, T.S. Dermody, Cathepsin L and cathepsin B mediate reovirus disassembly in murine fibroblast cells, J. Biol. Chem. 277 (27) (2002) 24609–24617, https://doi.org/10.1074/jbc.M201107200.
[55] A.U. Barlan, P. Danthi, C.M. Wiethoff, Lysosomal localization and mechanism of membrane penetration influence nonenveloped virus activation of the NLRP3 inflammasome, Virology 412 (2) (2011) 306–314, https://doi.org/10.1016/j.virol.2011.01.019.
[56] H.S. Krovi, L. Gapin, Structure and function of the non-classical major histocompatibility complex molecule MR1, Immunogenetics 68 (2016) 549–559, https://doi.org/10.1007/s00251-016-0939-5 [Online]. Available:.
[57] K.J. Goodall, A. Nguyen, A. Matsumoto, J.R. McMullen, S.B. Eckle, P. Bertolino, L. C. Sullivan, D.M. Andrews, Multiple receptors converge on H2-Q10 to regulate NK and γδT-cell development, Immunol. Cell Biol. 97 (3) (2019) 326–339, https://doi.org/10.1111/imcb.12222 [Online]. Available:.
[58] M. Peled, M.A. Dragovich, K. Adam, M. Strazza, A.S. Tocheva, I.E. Vega, A. Mor, EF hand domain family member D2 is required for T cell cytotoxicity, J. Immunol. 201 (9) (2018) 2824–2831, https://doi.org/10.4049/jimmunol.1800839.
[59] J. Wang, Y. Zhou, D. Li, X. Sun, Y. Deng, Q. Zhao, TSPAN31 is a critical regulator on transduction of survival and apoptotic signals in hepatocellular carcinoma cells, FEBS Lett. 591 (18) (2017) 2905–2918, https://doi.org/10.1002/1873-3468.12737.