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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome analysis of rainbow trout in response to non-virion (NV) protein of viral haemorrhagic septicaemia virus (VHSV)

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Abstract The non-virion (NV) protein of viral haemorrhagic septicaemia virus (VHSV), an economically important fish novirhabdovirus, has been implicated in the interference of some host innate mechanisms (i.e. apoptosis) in vitro. This work aimed to characterise the immune-related transcriptome changes in rainbow trout induced by NV protein that have not yet been established in vivo. For that purpose, immune-targeted microarrays were used to analyse the transcriptomes from head kidney and spleen of rainbow trout (*Oncorhynchus mykiss*) after injection of recombinant NV (rNV). Results showed the extensive downregulation (and in some cases up-regulation) of many innate and adaptive immune response genes not related previously to VHSV infection. The newly identified genes belonged to VHSV-induced genes (*vigs*), tumour necrosis factors, Toll-like receptors, antigen processing and presentation, immune co-stimulatory molecules, interleukins, macrophage chemotaxis, transcription factors, etc. Classification of differentially downregulated genes into rainbow trout immune pathways identified *stat1* and *jun/atf1* transcription factor genes as the most representative of the multipath gene targets of rNV. Altogether, these results contribute to define the role and effects of NV in trout by orchestrating an immunosuppression of the innate immune responses for favouring viral replication upon VHSV infection. Finally, these transcriptome results open up the possibility to find out new strategies against VHSV and better understand the interrelationships between some immune pathways in trout.

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Keywords Novirhabdoviruses · VHSV · NV · Transcriptome · Rainbow trout · Targeted-microarrays · *stat1* · *jun* · *atf1* · Interferon · *mx* · Caspases · TLR · VHSV-induced genes (VIG) · Interleukins · Chemokines · Complement · Macrophage · *tnf* · Antigen presentation machinery

Introduction

Viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), *Snakehead rhabdovirus* (SHRV) and *Hirame rhabdovirus* (HIRV) are species of a group of enveloped negative-stranded RNA rhabdoviruses affecting fish. They all have a single RNA genome of ~11 Kb (Nishizawa et al. 2002; Schütze et al. 1995, 1999) encoding five virion proteins (N, P, M, G and L proteins) and a non-virion (NV) protein. The presence of the NV gene was the common characteristic that grouped these viruses into the *Novirhabdovirus* genus (ICTV 2009), in contrast to other fish rhabdoviruses which lack NV, such as spring viraemia carp virus (SVCV). Novirhabdoviruses importantly affect wild-type and farmed fish. For instance, VHSV was isolated from more than ~50 fish species from North America, Asia and Europe, including ~15 farmed (Skall et al. 2005) and free-living marine fish species (Brudeseth and Evensen 2002). Some recent outbreaks happened in Asia, and Northern and Eastern Europe. Olive flounder was affected in Japan (Isshik et al. 2001) and Korea (Kim et al. 2009) where the latest outbreaks reported were in 1991 and 2005, respectively. For rainbow trout, some farms had outbreaks in Norway (2007) (Dale et al. 2009), Finland (2004) (Raja-Halli et al. 2006) and Slovenia (2007), where VHSV was eradicated since 1997 (Toplak et al. 2010). Finally, a North American novel strain

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has been isolated more recently (Pierce and Stepien 2012). VHSV is a notifiable disease to the Office International des Epizooties (OIE, Paris, France). The presence of VHSV in a fish farm leads to the sacrifice of all fish. However, it is difficult to add an economic estimation which really reflects this situation (Wahli and Bergmann 2011).

The NV gene is ~500 nucleotides in length, corresponding to 12- or 14-kDa proteins in IHNV or VHSV, respectively (Kurath et al. 1997). Despite of the NV gene presence in the four rhabdovirus species mentioned above, their NV proteins showed high homology intraspecies but very divergent interspecies sequences (Einer-Jensen et al. 2005; Kurath et al. 1997). Regarding their functionality, while NV was not essential for in vitro or in vivo SHRV production on warm-water flatfish (Alonso et al. 2004), it was required for highest efficient replication of IHNV (Biacchesi et al. 2010; Thoulouze et al. 2004) or VHSV (Kim and Kim 2012) on cold water rainbow trout or olive flounder, respectively. Comparative studies between wild-type (wt) and NV knockout IHNV or VHSV suggest that the NV protein may downregulate host *ifn1/mx* transcriptional levels during in vitro infection of rainbow trout gonad cells (RTG-2) (Choi et al. 2011) or cyprinid cells (EPC) (Kim and Kim 2012), respectively. Furthermore, interferons (IFN)-induced *mx* transcript levels were higher in NV knockout VHSV than in wt-VHSV-injected flounder, suggesting that NV interferes also in vivo with IFN defences to favour VHSV replication (Kim and Kim 2012). It has been also proposed that VHSV suppresses TNF-α-mediated NF-κB activation through NV in vitro (Kim and Kim 2013) and that NV plays an anti-apoptotic function as detected by downregulation of caspase 3, 8 and 9 activities (Ammayappan and Vakharia 2011).

Rainbow trout (*Oncorhynchus mykiss*) is one of the most economically important affected species by novirhabdoviruses such as VHSV. On the other hand, recent data from rainbow trout genomic/transcriptomic studies have increased the number of immune-related messenger RNA (mRNA) sequences available, making it possible the design of immune-targeted microarrays. Classification of the microarray probes into their immune pathways allowed for additional analysis of those genes common to several pathways (multipath genes), as used to study immune responses of zebrafish (*Danio rerio*) to infection with SVCV (Encinas et al. 2013). Because NV may be implicated in modulating more immune-related responses than those mentioned above, we carried out this study to identify the NV-driven transcriptional changes in rainbow trout, by using immune-targeted microarrays (Ballesteros et al. 2012). Since most studies up to date have compared wt- and NV knockout rhabdoviruses despite their different replication rates (~10^5 lower viral titers were obtained in vitro with NV knockout VHSV) (Biacchesi et al. 2010), a novel approach by using recombinant NV (rNV) have been employed here.

In trout, we found that rNV could play to favour viral replication through the downregulation (and upregulation) of a wide variety of innate immune genes and other regulatory genes not described before such as Toll-like receptors (TLRs), interferons, chemokines, interleukins, antigen presentation molecules, macrophage-related, and transcription factors. The expression of some adaptive immune genes is also downregulated. Multipathway gene analysis of downregulated genes suggests that *stat1* and *jun/atf1* genes encode for transcription factors (TFs) that are involved in more signalling pathways and could be inducing the observed wide effects. This work contributes to identify the role of NV in orchestrating a high variety of host downregulation of genes (some upregulated), coding for intracellular, intercellular and humoral proteins with innate and adaptive immune-related functions.

Materials and methods

Preparation of VHSV NV recombinant protein (rNV)

The DNA sequence corresponding to the NV protein of VHSV-07.71 strain isolated from rainbow trout (LeBerre et al. 1977) was used to obtain recombinant NV (rNV, GenBank accession number n° AJ233396). The corresponding NV sequence flanked by *Bam*HI and *Xho*I restriction enzyme sequences was synthesised (Bio S&T Inc., Montreal, Canada) and subcloned into the pRSETa plasmid (Invitrogen™, Life Technologies, Madrid, Spain) that contains a poly histidine tail (polyH) at the 5′-end (N-terminal) for protein purification. The pRSETa-NV construct confirmed by sequencing was used to transform *Escherichia coli* DH5α (Invitrogen™, Life Technologies, Madrid, Spain) by electroporation. High purity plasmid was prepared from *E. coli* pellets by using a modification of a commercial DNA purification system (Promega, Madison, USA). The DNA concentration was then estimated by Nanodrop ND1000 spectrophotometry measurements (Nanodrop Technologies Inc., Wilmington, DE, USA).

For expression of the rNV in bacteria, *E. coli* BL21 DE3 cells (Invitrogen™, Life Technologies, Madrid, Spain) were transformed with the pRSETa-NV plasmid, and the protein was expressed and purified following procedures previously described (Rocha et al. 2002). Western blot analysis of soluble and precipitated rNV was performed with anti-6× histidine monoclonal antibody (MAb) (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) and developed by enhanced chemiluminescence (ECL) as detailed before (Encinas et al. 2011). Numerous unsuccessful attempts were carried out to remove the amino terminal polyH tail of 33 amino acids (4 kDa, MRGSHHHHHHGMASMTGGQQMGRDLYDDDKDRW), included digestions with different proteases, solid-phase digestion methods and use of other cloning vectors and/or

purification procedures (i.e. Profinity eXact™ pPAL vector from Bio-Rad, Madrid, Spain). The so-called polyH tail was chemically synthesised and used as control to compare with rNV.

Purified rNV did not contain residual LPS (below 0.1 EU/ml) as determined by the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA).

Ethics statement

All procedures used in animals were approved by the ethical and biosecurity committee from INIA (authorisation CEEA 2011/022) and were performed following the National and European Commission guidelines and regulations on laboratory animals' care. All efforts were made to minimise animal suffering. Fingerling rainbow trout were anaesthetised by immersion in 50 mg/ml tricaine methanesulfonate (MS-222, Sigma, Madrid, Spain) and sacrificed by decapitation.

Intracellular incorporation of rNV by rainbow trout head kidney cells

To estimate intracellular incorporation of soluble rNV by rainbow trout head kidney cells, both in vivo and in vitro assays were performed. Thus, while for the in vivo assays, trout were injected with rNV (1 μg of rNV/g of trout) and head kidney cells extracted to be analysed by flow cytometry, for the in vitro assays head kidney cells were first extracted and then incubated with rNV (1–50 ng of rNV per well) before flow cytometry. Cell sorting from head kidney intended to differentiate lymphocytes, macrophages and erythrocytes and if any of these populations acquired the rNV protein. Thus, head kidneys were removed from rainbow trout and their cells isolated by passing the tissue several times through 27G and 30G needles. The cellular suspensions were prepared for in vitro culture by washing with cell culture medium. After being counted with an haemocytometer, the cells were plated (200,000 round cells per well) in a final volume of 100 μl in poly-D-Lys 96-well plates (Corning, NY, USA) and rNV at 5 to 50 ng per 100-μl concentrations added. Plates were incubated overnight at 14 °C in a 5 % CO_2 atmosphere.

In both in vivo and in vitro assays, to stain for intracellular rNV, the cells were first fixed to the solid-phase of the plates by following our published procedure (Chinchilla et al. 2013). For detecting rNV, an anti-6× histidine MAb (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) was added to the wells 500-fold diluted in 2 % FCS, 0.05 % saponin and 0.01 % N_3Na in PBS (permeabilising buffer) and incubated for 60 min. After washing with the permeabilising buffer and manually flicking the plate, rabbit FITC-labelled anti-mouse IgG (Nordic MUbio, Susteren, The Netherlands) was added and incubated for 30 min. After washing with non-permeabilising buffer (same as before but with no saponin), the monolayers were incubated 10 min with 100 μl of 0.25 % trypsin 0.02 % EDTA (Sigma-Aldrich Quimica SA, Madrid, Spain) with strong agitation to suspend the cells for flow cytometry. Trypsin digestion was stopped by the addition of 50 μl per well of 1 % BSA, 50 mM EDTA, 0.01 % N_3Na in PBS and well contents pipetted up and down several times until most cells were suspended. The suspended cells were then analysed in a BD FACSCanto II apparatus (Beckton Dickinson, Madrid, Spain) provided with a high throughput sampler (HTS). Forward (FSC) and side (SSC) scatter threshold values corresponding to damaged cells, cellular debris and/or cellular aggregates (<10 % of total events) were eliminated from the analysis. Cells were gated for FSC/SSC populations (P1–P5) and the number of fluorescent cells in each population over a threshold containing 95 % (mean+2 standard deviations) of non-rNV-treated cells was then determined (Chinchilla et al. 2013; Ruiz et al. 2008). After the multiwell autosampler gated 10,000 events per well of the 96-well plates (60 min), the automatic batch analysis feature of BD FACSDiva calculated the percentage of fluorescent (rNV-positive) cells by the formula 100×number of cells in each population with fluorescence values above the fluorescence threshold/total number of cells gated per population per well. The software exported results to an MS Excel sheet for additional calculations, and those were plotted in Origin Pro 8.6 (OriginLab Corporation, Northampton, MA, USA). Comparisons of percentage of fluorescent cells were performed by using the Student's t test at the $p \leq 0.05$ level. The anti-6× histidine MAb-stained cells were stained with TO-PRO®-3 (Molecular Probes®, Life Technologies, Madrid, Spain), observed and photographed using a Leica DC100 confocal microscope (Leica Microsistemas S.L.U., Barcelona, Spain).

Injection of fingerling rainbow trout with rNV

Three different batches of fingerling rainbow trout (*O. mykiss*) of ~10 g each, free of IPNV and VHSV antibodies, were obtained from a local fish farm (Los Molinos, Madrid, Spain). Trout from different batches were pooled and maintained at 14–15 °C in 200-l aquaria with tap-dechlorinated carbon-filtered water provided with biological filters and fed with a commercial fish feed diet. After 2 weeks of acclimation, trout were separated into groups of six to eight trout per group and intraperitoneally injected with soluble rNV (10 μg), precipitated rNV (10 μg), polyH (2 μg, which is equivalent in moles to rNV) or PBS in a volume of 100 μl. Injected trout were then released to a 50-l aquarium per group and maintained at 14 °C. Two days after injection, trout were anaesthetised as described above. Pooled head kidney and spleen from each trout were extracted, immediately immersed in RNAlater (Ambion®, Life Technologies, Madrid, Spain) and maintained at 4 °C overnight before being frozen at −70 °C until processed. Ten micrograms of rNV per trout were intraperitoneally injected

because this amount is ~10-fold higher than the amount of NV estimated in a VHSV-infected 10-g trout. The calculations were based on published estimations of number of active plaque-forming unit (pfu) per gram of trout (LaPatra et al. 1995), number of N molecules per pfu (Coll 1995), number of VHSV particles per pfu (Leong 1995), percentage of defective particles (Kim et al. 1999) and relative amounts of NV/N proteins in Western blots (Schütze et al. 1996). In addition, the NV/N ratio estimated by reverse transcription quantitative PCR (RT-qPCR) was 0.06, 2 days after intraperitoneal injection of 10^4 pfu of VHSV (not shown).

RNA extraction

Pooled head kidney and spleen from each individual trout were homogenised by using the Tissue Lyser Cell Disruptor (Qiagen Iberia, S.L., Madrid, Spain) 10 min at 50 Hz with 3-mm glass beads in RTL buffer. RNA was then extracted from the homogenates by using the RNAeasyPlus kit (Qiagen Iberia, S.L., Madrid, Spain) and eluted in RNase-free water. RNA concentrations were estimated by Nanodrop and the presence of 18 and 28S bands confirmed by denaturing RNA agar electrophoresis (Sigma-Aldrich Quimica SA, Madrid, Spain). Additional RNA quality control (RNA integrity number, RIN) was performed by NIMGenetics (Madrid, Spain). For each experimental group of six to eight trout, four of the best quality RNA (RIN>7.0) per group were chosen for microarray hybridisation. The number of biological replicas was 4, one trout per replica. Head kidney and spleen were pooled from each individual trout. Each biological replica was hybridised twice (technical replicas). The total number of chips used was 4 (eight samples per slide), and all were hybridised simultaneously.

Design of oligo-microarrays enriched in rainbow trout immune-related genes (targeted microarrays)

The microarrays were enriched in rainbow trout immune-related genes as described previously (immune-targeted microarrays) (Ballesteros et al. 2012). The final 8x15K microarray corresponds to Agilent's ID032303 (Gene Expression Omnibus GEO platform submission number GPL14155). It contains 60-mer annotated probes each by duplicate, and different probes could detect the expression changes for each gene. To simplify the analysis of results, 1474 annotated probes were classified according to gene groups: VHSV-induced genes (VIG; number of probes, n=26), interferons (IFN) and its receptors (n=92), interferon-inducible proteins *mx* (MX; n=8), Toll-like receptors (TLR; n=32), complement components (CO, n=177), interleukins and its receptors (IL; n=120), macrophage-related genes (MA; n=126), tumour necrosis factor (TNF; n=33), cluster differentiation (CD; n=59) antigens, chemokines (CK) and its receptors (n=122), and caspases (8) (CASP) and transcription factors (TFs) (n=672). The trout microarray ID32303 used for these experiments was previously validated by RT-qPCR (Ballesteros et al. 2012). The number of biological replicas was 4, one trout per replica. Head kidney and spleen were pooled from each individual trout. Each biological replica was hybridised twice (technical replicas). The total number of chips used was 4 (eight samples per slide), and all were hybridised simultaneously. Although to discover other possible non-immune genes targeted by rNV, a complementary DNA (cDNA) genome-wide microarray (e.g. 8x60K Salmonid from Agilent) could be more complete, we chose to focus on immune genes because there are no oligo microarrays available with such an enrichment of rainbow trout immune genes, there exist many non-annotated genes in available salmonid microarrays, and cDNA salmonid microarrays have higher crossreacting hybridisations and more qPCR is needed to validate the data. Our home-made rainbow trout oligo microarray used has more immune genes than any other available microarrays since it includes all the immune-related genes from the Agilent's EST-derived rainbow trout oligo microarray (ID16271) (Salem et al. 2008) as described before (Ballesteros et al. 2012).

Hybridisation of trout transcripts to the immune-targeted microarrays

Labelling of 2 μg of RNA (~50 μg/ml) and hybridisation to the microarrays were performed by NIMGenetics (Madrid, Spain) complying with the Minimum Information about a Microarray Experiment (MIAME) standards (Ballesteros et al. 2012).

Normalisations were performed by correcting the individual fluorescence in each microarray with the sum of all the fluorescent values according to the formula fluorescence of each probe/sum of all the probe fluorescence signals per microarray. The raw and normalised data were deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37330). After normalisation, outlier values (defined by those fluorescence values > or < mean±standard deviation per probe) were identified and eliminated (masked) from the calculations programmed in Origin Pro 8.6 (OriginLab Corporation, Northampton, MA, USA). Corrections with PBS (mock injected controls) values were first calculated for each probe as reported previously (Purcell et al. 2006) by using the following formula fluorescent value from rNV- or polyH-injected trout/mean fluorescent value from PBS-injected trout for obtaining rNV/PBS and polyH/PBS datasets, respectively. Final folds represented by rNV/polyH were then calculated by the following formula PBS corrected values from rNV-injected trout (rNV/PBS)/mean PBS-corrected value from polyH-injected trout (polyH/PBS). Means and standard deviations of the folds were then calculated (n=4). The two-tailed

Student's *t* test statistic-associated *p* value was also computed for each probe. A double simultaneous criterion to define differentially expressed gene transcripts was used: (i) genes with folds >1.5 or <0.66 (drawn in the figures as negative values <−1.5) and (ii) folds deviated from the null hypothesis at $p \leq 0.05$.

Classification of rainbow trout genes in immune pathways and identification of genes common to many pathways (multipath genes)

To classify the rainbow trout genes of the ID032303-targeted microarray into immune-related pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/) and the WikiPathways (http://wikipathways.org/index.php/WikiPathways) pathway databases were used. Rainbow trout orthologous to human genes from available 74 immune pathways were manually searched on GenBank mRNA sequences (accessed from February to June of 2013). The list of the rainbow trout genes classified by pathways and their accession numbers were deposited in the GEO platform with submission number GPL17758. Each of the genes of the ID032303 microarray was then classified into the above-mentioned rainbow trout pathways by using a macro in Excel. To test for differentially expressed multipath genes, microarray genes were first classified into 74 rainbow trout immune-related pathways. In order to do that, the list of accession numbers of the microarray genes was first compared with the rainbow trout accession numbers obtained by manually searching their corresponding orthology in immune-related human pathways actually present in public databases. The classified genes were then systematically searched for genes (i) common to at least six immune-related pathways (Encinas et al. 2013), (ii) with differential expression folds <0.66 or >1.5 and (iii) significantly different from one of the 0.66/1.5 thresholds ($p \leq 0.05$). The pathways having more than five differentially expressed multipath genes were identified by using a program made in Origin Pro 8.6 (OriginLab Corporation, Northampton, MA, USA).

Results

Purification and yield of rNV

The protein extracts from recombinant *E. coli* coding for rNV were purified under denaturing conditions by His-tag affinity chromatography (IMAC) and eluted by imidazole yielding a single protein peak. Further chromatography on Sephadex G-100 at low pH and final dialysis against PBS at pH 7 produced abundant precipitates with only 4–7 % of the total rNV protein remaining in solution. Western blot analysis of soluble rNV with an anti-6× histidine monoclonal antibody (MAb) showed one band of ~17 kDa (Fig. 1). Numerous attempts to remove the amino terminal polyH tail of 33 amino acids were only capable of removing less than 55 % of the polyH tail (data not shown). Therefore, equimolecular amounts of a synthetic polyH peptide of 33 amino acids (4 kDa, MRGSH HHHHHGMASMTGGQQMGRDLYDDDKDRW) were injected also into trout as a control to derive differential expression data. Both resuspended-diluted precipitate and soluble supernatant were used to inject trout.

Effects on trout transcriptome modulation produced by rNV could not be due to LPS contamination since the amount in purified rNV was below 0.1 EU/ml.

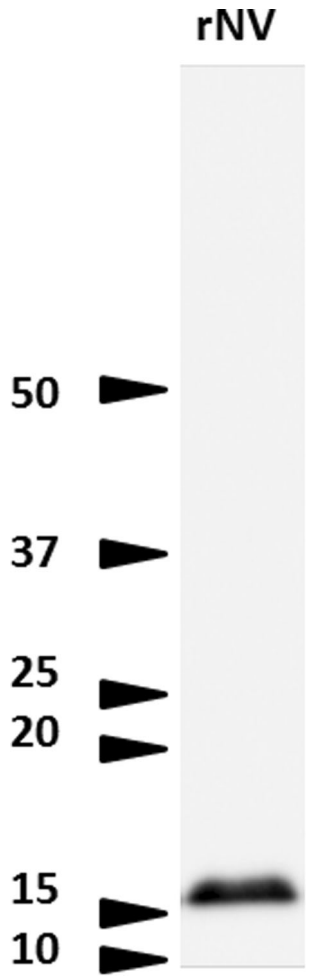

Fig. 1 Detection of rNV by Western blot. The nucleotide sequence of recombinant NV (*rNV*) derived from the VHSV-07.71 strain was cloned into the *E. coli* pRSETa plasmid. The rNV was purified in denaturing conditions by a Ni^{2+} affinity column and eluted with imidazole as indicated in 'Materials and methods'. Soluble rNV was separated by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and reacted with poly-6× histidine monoclonal antibody. The numbers to the left correspond to molecular weight standards in kilodalton

Selection of delivery route

Despite NV being an intracellular VHSV-expressed protein only in infected cells, extracellular NV should be also released after cellular lysis, as natural VHSV infection progresses from the point of infection. To study the possibility of extracellular NV being taken by some rainbow trout cells, head kidney cells were incubated in the presence of several concentrations of soluble rNV and its possible intracellular incorporation analysed by flow cytometry. Results demonstrated that 4–9 % of the cells in the P4 cell population (one of the populations containing 9.7±2.2 % of the total cells) and 2–5 % of the cells in P5 significantly incorporated extracellular rNV (Fig. 2a). The level of incorporation increased with rNV concentration, being highest at >50 ng/100 μl (Fig. 2b). Therefore, rNV could be injected into trout to allow for either soluble and/or macrophage-carried rNV to target internal immune-responsive organs such as head kidney and spleen. We chose intraperitoneal (ip) injection as the most practical delivery route to induce possible rNV effects on head kidney and spleen and because of the presence of peritoneal melanomacrophages in trout. Thus, head kidney and/or spleen cells isolated from fingerling trout ip injected with 1 μg of soluble rNV per gram of fish showed similar staining properties by flow cytometry analysis (Fig. 2c) than those obtained in the in vitro assays mentioned above. The ip injection of rNV could be a good approach for studying the NV effects on rainbow trout head kidney and spleen because VHSV naturally infects cells from these organs. Confirming the above-mentioned observations, the only cellular type which appeared with green fluorescence (detecting the presence of poly-6× histidines) at the confocal microscopy in both in vitro and in vivo experiments were typical melanomacrophages as demonstrated by their morphology. Melanomacrophages (n=50) took up rNV in their cytoplasm (Fig. 2d, fluorescence in green) rather than in its nuclei (Fig. 2d, fluorescence in blue) as shown by confocal microphotographs (Fig. 2c). Cytosolic location of rNV was previously observed in ZF4 and EPC-transfected cells with plasmid expressing His-tagged rNv and detected by fluorescence confocal microscopy (data not shown). No other cellular types showed green fluorescence.

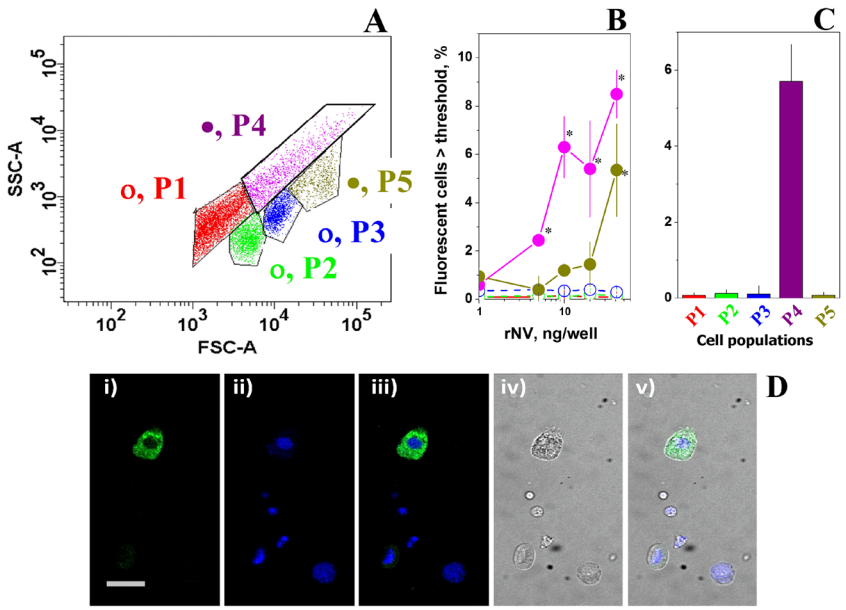

Fig. 2 Fluorescent cells in rainbow trout head kidney cell populations after in vitro incubation or in vivo injection of rNV. Head kidney cells were extracted from fingerling rainbow and separated by flow cytometry. **a** Five SSC/FSC cell populations were defined: *P1* in *red* (50.3±10.4 % of the total number of head kidney cells), *P2* in *green* (12.4±5.4 %), *P3* in *blue* (10.7±4.0 %), *P4* in *violet* (9.7±2.2 %) and *P5* in *dark yellow* (3.8±2.7 %). **b** Cells were plated (200,000 round cells per well) in a final volume of 100 μl in poly-D-Lys 96-well plates (Corning, NY, USA), soluble rNV added at different concentrations (1–50 ng/well) and plates incubated overnight at 14 °C before analysis. **c** Trout of ~1 g were injected with 1 μg of soluble rNV and 1 day later head kidney cells extracted. In both **b** and **c**, to detect intracellular rNV, cells were fixed to the plates, stained with anti-6× histidine MAb and suspended for flow cytometry by limited trypsin digestion (Chinchilla et al. 2013). **b**, **c** Distribution of the percentage of fluorescent cells among *P1–P5* cell populations. The number of fluorescent cells over a threshold containing 95 % (mean+2 standard deviations) of untreated cells was determined (Chinchilla et al. 2013; Ruiz et al. 2008). Mean and standard deviations were represented (n=2 for **b** and n=6 for **c**). *Significantly different from untreated cells by using the Student's t test at the $p \leq 0.05$ level. ○ *red*, P1.○ *green*, P2. ○, *blue*, P3. ●, *violet*, P4.●, *dark yellow*, P5. **d** Cytosolic location of rNV and cell morphology revealed by confocal microscopy. From *left* to *right*: green fluorescence locates rNV, detected by anti poly-histidine monoclonal antibody (*i*), blue fluorescence, TO-PRO-3-stained nuclei (*ii*), merged green and blue fluorescence (*iii*), phase contrast (*iv*) and merged all fields (*v*). *White horizontal bar*, 15 μm (colour figure online)

Overview of microarray results after intraperitoneal injection of rNV

To choose a suitable control to study the effects of rNV injection, the hybridisation to microarrays of RNA from head kidney and spleens from trout injected with polyH vs PBS was first studied. The analysis showed that 14.5 % of the probes were significantly expressed suggesting that injection of polyH had a small but measurable effect on trout transcript expression. Notwithstanding, this synthetic peptide did not induce the upregulation or downregulation of *mx*, *tlr*, *ifn* or *isg* genes, when compared with the PBS transcriptome profile. Therefore, polyH rather than PBS should be used to derive differentially expressed transcripts for polyH containing rNV.

The levels of transcription induced by the injection of soluble or precipitated rNV were each compared to PBS and then each compared to those caused by the injection of 2 μg polyH (corresponding to an equimolecular amount of that present in rNV). Most of the fluorescence signals from soluble rNV vs polyH-injected trout were <1-fold (down the black line in Fig. 3). In contrast, fluorescence signals from precipitated rNV vs polyH injected trout were more evenly distributed around fold 1 (not shown). Thus, the comparative analysis of both soluble or precipitated rNV vs polyH showed 52.8 or 8.5 % differentially expressed genes, respectively (data deposited in GSE 31557 and 37797), suggesting that rNV was required in soluble form to obtain maximal downregulation. The small amount of downregulated genes after injection of precipitated rNV provides a suitable specificity control for the soluble rNV effects. Therefore, soluble rNV was used to analyse more in detail differential expressions induced in each of the genes grouped in the categories described in 'Materials and methods'.

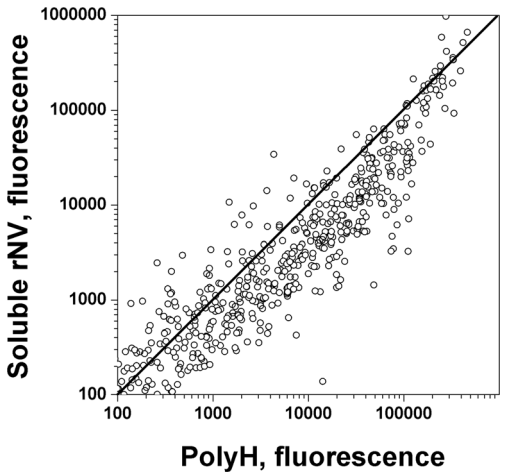

Fig. 3 Microarray fluorescence signals obtained from rainbow trout injected with soluble rNV. The figure shows the fluorescence signals of the transcriptomic profiles induced by soluble rNV vs polyH. Trout were injected with 10 μg of soluble rNV or 2 μg of polyH synthetic peptide (33 aa). The number of biological replicas was 4, one trout per replica. Head kidney and spleen were pooled from each individual trout. Each biological replica was hybridised twice (technical replicas). The total number of chips used were 4 (eight samples per slide) and all were hybridised simultaneously. After normalisation and outlier removal, the figure shows the fold means from four trout from each group. The fluorescence signals >100 arbitrary units (background fluorescence) were plotted. In each case, a *black straight line* has been drawn to show folds =1

Downregulation of IFN, myxovirus resistance and CASP by rNV. Novel and confirmatory results

The differential expression of selected genes known to be downregulated by wt-VHSV when compared to NV knockout VHSV such as interferon-related genes (i.e. type I interferons *ifn1-5* and interferon gamma, *ifng*) (De Kinkelin and Dorson 1973), *mx* (*mx1–mx3*) (Tafalla et al. 2007) and genes for caspases (Ammayappan and Vakharia 2011) was first studied. In this work, we have found multiple probes of type-1 interferon genes (*ifn1-5*) and *ifng* downregulated by rNV, confirming those previous reports. In addition, novel *ifn*-inducible genes (i.e. *gig2*, *iip2*, *iip30*, *ipf35*) and many *ifn* regulatory binding genes (*irf2*, *irf4*, *irf7*, *irf10*) were also downregulated (Fig. 4, IFN). Downregulation induced by rNV was similarly observed in all the myxovirus resistance GTPase genes (*mx1–mx3*) together with a gene for another GTPase regulator of G protein signalling (*rgs18*) (Fig. 4, MX). Caspase transcripts such as *casp1a*, *casp9* (initiator caspase) and *casp6* (effector caspase) were downregulated also by rNV injection (Fig. 4, CASP). Slight downregulation was found also for *casp3* (effector caspase). The complete names of the genes shown in the figures have been included in the legends.

Novel effects of rNV on the expression of TFs

Downregulated genes by rNV among *stat* (signal transducer and activator) factors were *stat1* (involved in IFN-α/β and IFN-γ signalling pathways), *stat3* (negative regulator of COX2/prostaglandin E), and *stat5* (Fig. 4, TF). No expression data could be obtained for other *stat* transcripts since they have not been described in trout yet. Other downregulated genes were *nfkb* (p100/p52), *traf3* (tumour necrosis factor receptor-associated factor 3), *cebp* (ccaat/enhancer-binding protein), *atf1* (cAMP-dependent transcription factor), *jun* (proto-oncogen) and *tr* factor. Because the activating protein 1 (AP1, heterodimer composed by JUN, FOS, ATF1 family proteins), CEBP, STAT1 and TRAF2 proteins control the expression of many immune response genes (i.e. cytokines, interferons, interleukins, regulatory factors, etc), their gene downregulation should lead to a general inhibition of immune responses. The complete names of the genes shown in the figures have been included in the legends.

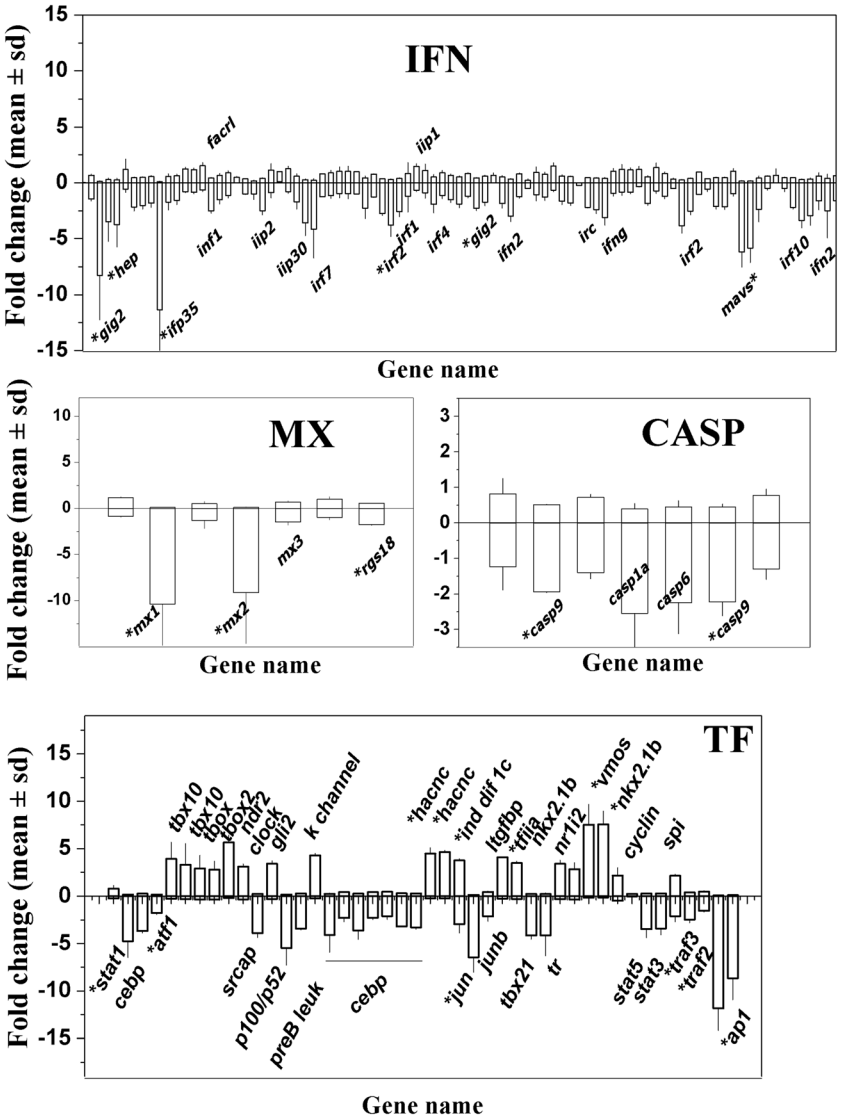

Fig. 4 Transcriptional expression changes induced by rNV among interferon (*IFN*) genes, myxovirus resistance genes (*MX*), caspase (*CASP*) genes and transcription factor (*TF*) genes. Differential fold changes±standard deviations are represented by bars (*Y*-axis). Probes defining distinct genes are represented in *X*-axis. For each probe, differential expression folds were first calculated by comparing profiles from rNV or polyH-injected trout with respect to PBS-injected trout controls by the formula fluorescence from rNV or polyH injected trout/mean fluorescence of PBS injected trout controls (n=4). Final folds were then calculated by the formula PBS corrected values from rNV-injected trout/mean PBS-corrected value from polyH-injected trout (n=4 trout). *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). IFN group, genes upregulated: *facrl*=interferon regulatory factor-like and *iip1*=interferon inducible protein 1 (AJ320156). Genes downregulated: *hep*=hepcidin, *gig2*=Tribbles homolog, G-protein-coupled receptor-induced gene 2 protein, *ifn1*=type 1 interferon 1, *ifn2*=type 1 interferon 2, *ifng*=interferon gamma, *ifp35*=interferon-induced protein 35, *iip2*=interferon inducible protein 2, *iip30*=interferon gamma inducible protein 30, *irc*=insulin receptor c, *irf1*=interferon regulatory factor 1, *irf2*=interferon regulatory factor 2, *irf4*=interferon regulatory factor 4, *irf7*=interferon regulatory factor 7, *irf10*=interferon regulatory factor 10 and *mavs*=mitochondrial antiviral signalling protein. MX group, genes downregulated: *mx1*=myxovirus resistance 1, *mx2*=myxovirus resistance 2, *mx3*=myxovirus resistance 3 and *rgs18*=regulator of G-protein signalling 18. CASP group, genes downregulated: *casp9*=caspase 9, *casp1a*=caspase 1a and *casp6*=caspase 6. TF group, genes upregulated: *clock*=circadian locomoter output cycles kaput protein, *cyclin*=cyclin, *gli2*=zinc finger transcription factor gli2, *hacnc*=hyperpolarisation-activated cyclic nucleotide-gate cation channel 2, *ind dif 1c*=inhibitor differentiation factor 1c, *K channel*=potassium channel tetramerisation domain, *ltgfbp*=latent transforming growth factor binding protein, *ndr2*=serine/threonine kinase 38 like, *nkx2.1b*=nk2 homeobox 1b, *nr1i2*=nuclear receptor subfamily 1, group I, member 2, *spi*=SPI-1 transcription factor, *tbox*=T-box containing transcription factor, *tbox2*=T-box 2 gene, *tbx10*=T-box 10 gene, *tfiia*=general transcription factor IIA and *vmos*=V-mos Moloney murine sarcoma viral onco-like prot. Genes downregulated: *ap1*=AP1 transcription factor, jun proto-oncogen, *atf1*=cAMP-dependent transcription factor ATF-1, *cebp*=CCAAT/enhancer binding prot, *jun*=jun proto-oncogen, *junb*=jun b proto-oncogen, *p100/p52*=p100/p52 transcription factor (*nf-kb2*), *preB leuk*=pre-B-cell leukemia transcription factor protein 1, *srcap*=helicase SRCAP gene, *stat1*=signal transducer and activator of transcription 1, *stat 3*=signal transducer and activator of transcription 3, *stat5*=signal transducer and activator of transcription 5, *tbx21*=T-box 21 gene, *tr*=tissue factor, initiator of coagulation cascade, *traf2*=tnf receptor-associated factor 2 and *traf3*=tnf receptor-associated factor 3

Novel downregulation by rNV on the expression of VIG, TNF and TLR genes

Surprisingly, all VHSV-induced genes (*vig*) identified by subtractive hybridisation in previous works (O'Farrell et al. 2002), except *b191*, were significantly downregulated by rNV (Fig. 5, VIG). Most of the genes for inducers of inflammation known as members of the tumour necrosis factor (*tnf*) family were also downregulated by rNV, including several probes of *tnf10* (apoptosis inducer acting through *casp3* and *casp8*), *tnf11* (dendritic survival factor and activator of naïve T cells), *tnf14* (stimulator of apoptosis), *tnfa* (most important inducers of systemic inflammation), *ltb2*, *balm* and several *tnf* receptors (*tnfr*) (Fig. 5, TNF). Similarly, most of the genes for Toll-like receptors (*tlr*) were downregulated by rNV. Thus, gene expression of *tlr1* (receptor for microbial lipopeptides and glycolipids), *tlr3* (receptor for short-size dsRNA in membranes), *tlr22* (receptor described in zebrafish and catfish that senses long-size dsRNA) (Baoprasertkul et al. 2007; Zhang et al. 2013), *tlr7* (receptor for ssRNA) and *tlr9* (sensing CpG DNA) were downregulated (Fig. 5, TLR). In addition, negative regulators *tollip1* and *tollip2* were downregulated. Contrary to the gene *tlr5* for a membrane TLR form that remained unchanged, the gene for a soluble form was upregulated (Fig. 5, TLR). The complete names of the genes shown in the figures have been included in the legends.

Novel effects of rNV on the expression of APM genes, CD antigens and IL

Most of the genes involved in antigen presentation machinery (APM) such as *tapbp* (codes for tapasin protein), *tap1* and *tap2* (transporter associated with antigen processing), *lmp2* (coding for a protein of the proteasome) and *b2m* (beta 2 microglobulin) were downregulated (Fig. 6, APM). Among the cluster differentiation (CD) antigens studied, not only downregulated but also upregulated genes were detected. Thus, some of the genes which were downregulated by rNV were *cd2* (implied in adhesion T cell-APC through CD58 protein), *cd3e* (part of the T cell receptor complex), *cd4* (T-helper cell marker), *cd8* (T-cytotoxic cell marker), *cd9* (thymocyte cell marker), *cd11* (dendritic cell marker, included in Fig. 7, MA), *cd28* (co-stimulatory gene expressed in T-cells), *cd80/86 m* (short mature form of a gene expressed in the membrane of antigen presenting cells), *cd79a* (associated with membrane-bound immunoglobulin in B-cells) or *cd200* (NK immunosuppressive cell surface gene) (Fig. 6, CD). In contrast, *cd33* (present in immature myeloid cells, possible Siglec-3 receptor), *cd97* (component of the immunoglobulin receptor of B cells, possibly related to decay accelerating factor of complement), *cd209* (marker of dendritic and macrophage cell subsets, which may increase phagocytosis) and *cd80/86 s* (soluble form of the gene) were upregulated (Fig. 6, CD).

Among the interleukin-related probes tested, there were also downregulated and upregulated genes. Thus, many of the interleukin genes (*il*) were downregulated, such as several probes of *il1b* (a lymphocyte mitogen produced by macrophages and an important mediator of inflammatory responses) and the gene for its receptor (*il1r*), *il2* (secreted by T cells), *il8* (a chemotactic factor produced by macrophages that attracts neutrophils, basophils and T-cells), *il12b* (maintenance of T helper responses to intracellular pathogens), *il15* (regulator of lymphoid homeostasis, effector of T cells and NK activation), *il17d* and its receptor (*il17r*). In contrast, other interleukin genes such as *il20* (regulator of proliferation/differentiation of keratinocytes), *il10* (an inhibitor of cytokines with anti-inflammatory effects), *il17a* and c (pro-inflammatory cytokine of activated T cells), *il18* and receptors like *il8r*, *il1r* or *il13r* (interleukin 13 (IL13), a pleiotropic cytokine involved in the regulation of IFNγ were upregulated (Fig. 6, IL). The complete names of the genes shown in the figures have been included in the legends.

Novel effects of rNV on the expression of macrophage cell markers (MA), CK and CO

Among the probes present in the microarray classified as macrophage-related genes, the genes for macrophage stimulating factor (*mcsf*), natural resistance macrophage α (*nrampa*), pre-B cell colony-enhancing factor (*pbef*), *cd11* (a dendritic cell marker) and cadherin (*cdh*) coding for an adhesive molecule (Fig. 7, MA) were among those downregulated by rNV. Only the genes for colony-stimulating factor 1 (*csf1*) and c-reactive protein (*crp*) were upregulated. Among the genes for chemoattractant cytokines or chemokines (*ck*) showing downregulated gene expressions after rNV injection were *ck1*, *ck2*, *ck3*, *ck5b*, *ck6*, *ck8b*, *ck10*, *ck12a*, *ccl4* (*scya4*), *cxc*, *cxcd1*, *cxcd2*, etc. Downregulation was also shown in some genes causing cytokine inhibition such as *socs1–socs7* (Fig. 7, CK). On the other hand, rNV induced upregulation of *ck4a*, *ck11*, *cxcr* and *ccr5* (Fig. 7, CK). The rNV downregulated many genes of the classic complement pathway, such as *c1q*, *cr1* (*c1* receptor), *c4* and perforin (*prf*). On the other hand, *bf1* and *h1* (inhibitors of complement) factors as well as some components of the final effector complex (*c5*, *c7*, *c8*, *c9*) were upregulated by rNV (Fig. 7, CO). The complete names of the genes shown in the figures have been included in the legends.

Identification of regulated genes common to several pathways (multipath genes) suggests functionally important NV targets

Because the above-mentioned analyses of microarray data were mainly descriptive, we analysed the differentially expressed genes based on their presence on rainbow trout/human orthologous immune pathways (multipath genes). Thus, the numerous immune-related genes downregulated by rNV

suggested that a few master genes targeted by NV could be responsible. Those candidates could be amongst multipath genes since they would interconnect several pathways and therefore have the widest impact. Differentially expressed multipath genes filling the above-mentioned criteria were thus identified (Table 1). Among all genes downregulated by rNV, there are three (*stat1*, *jun* and *atf1*) whose transcription factors are present in ≥20 immune-pathways and in some of the pathways containing the highest numbers of multipath genes such as for TLR (n=9 multipath genes), interferon type I (n=6), MAPK (n=6) and RIG-I (n=6). All the rest of the differentially expressed multipath genes identified were also downregulated

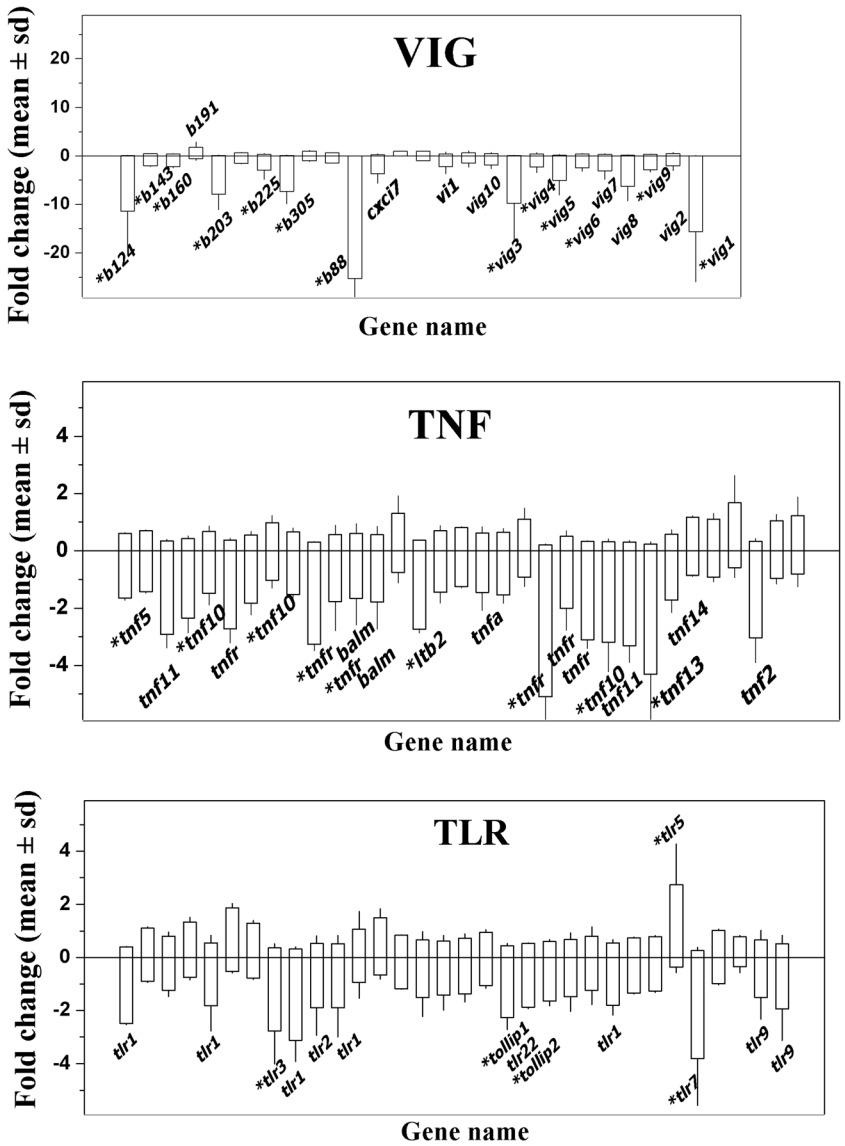

Fig. 5 Transcriptional expression changes induced by rNV among VHSV-induced (*VIG*), tumour necrosis factor (*TNF*) and Toll-like receptor (*TLR*) gene groups. Differential fold changes±standard deviations are represented by *bars* (*Y*-axis). Probes defining distinct genes are represented in *X*-axis. *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). Fold change calculations are described in 'Materials and methods' and Fig. 4 legend. VIG group, genes upregulated: *b191* (AF483535). Genes downregulated: *vig-1* (AF076620), *vig-2* (AF290477), *vig-3* (AF483529), *vig-4* (AF483530), *vig-5* (clone B17), *vig-6* (clone B126), *vig-7* (AF483527), *vig-8* (clone B68), *vig-9* (AF483533), *vig-10* (AF483534), *b203* (AF483538), *b143* (AF483539), *b225* (AF483540), *b88* (AF483541), *b160* (AF483545), *b124* (AF483546), *b305* (AF483542), *cxci7*=VHSV-induced protein 7 (*vig7*) and *vi1*=VHSV-induced protein 1. TNF group, genes downregulated: *balm*=BAFF and APRIL-like molecule, *ltb2*=lymphotoxin beta 2, *tnf10*=tumour necrosis factor superfamily 10 (TNFSF10), *tnf11*=tumour necrosis factor superfamily 11, *tnf13*=tumour necrosis factor superfamily 13, *tnf14*=tumour necrosis factor superfamily 14, *tnf2*=tumour necrosis factor superfamily 2, *tnf5*=tumour necrosis factor superfamily 5 (CD40), *tnfa*=tumour necrosis factor alpha and *tnfr*=tumour necrosis factor receptor. TLR group, genes upregulated: *tlr5*=Toll-like receptor 5. Genes downregulated: *tlr1*=Toll-like receptor 1, *tlr2*=Toll-like receptor 2, *tlr3*=Toll-like receptor 3, *tlr7*=Toll-like receptor 7, *tlr9*=Toll-like receptor 9, *tlr22*=Toll-like receptor 22, *tollip1*=Toll-interleukine I receptor interacting protein 1 and *tollip2*=Toll-interleukine I receptor interacting protein 2

after rNV injection (*traf3*, *traf2*, *tnfa*, *ifng*, *casp6*, *irf1*, *il1b*, *irf10*, *socs1*, *ifn1*, *irf2*) (Table 1).

Discussion

In the present work, we have determined the trout transcriptome changes induced by the recombinant NV (rNV) protein from VHSV after 48 h post-intraperitoneal injection. Previous published results showed that a natural VHSV infection on EPC cells induced the highest NV expression level at 48 h post-infection (hpi) (Kim and Kim 2012). Therefore, we decided to study the transcriptome changes at 48 h as the best approach to the natural conditions of infection. NV effects might be different on gene transcriptome modulation along time. However, we have taken two considerations: the highest NV expression at 48 hpi (Kim and Kim 2012) and possible half-life of rNV within cell. In mammals, GFP protein has half-life of ~26 h (Corish and Tyler-Smith 1999) and the precursor IL1β is degraded with a relatively short half-life of 2.5 h (Moors and Mizel 2000). Therefore, it is probable that

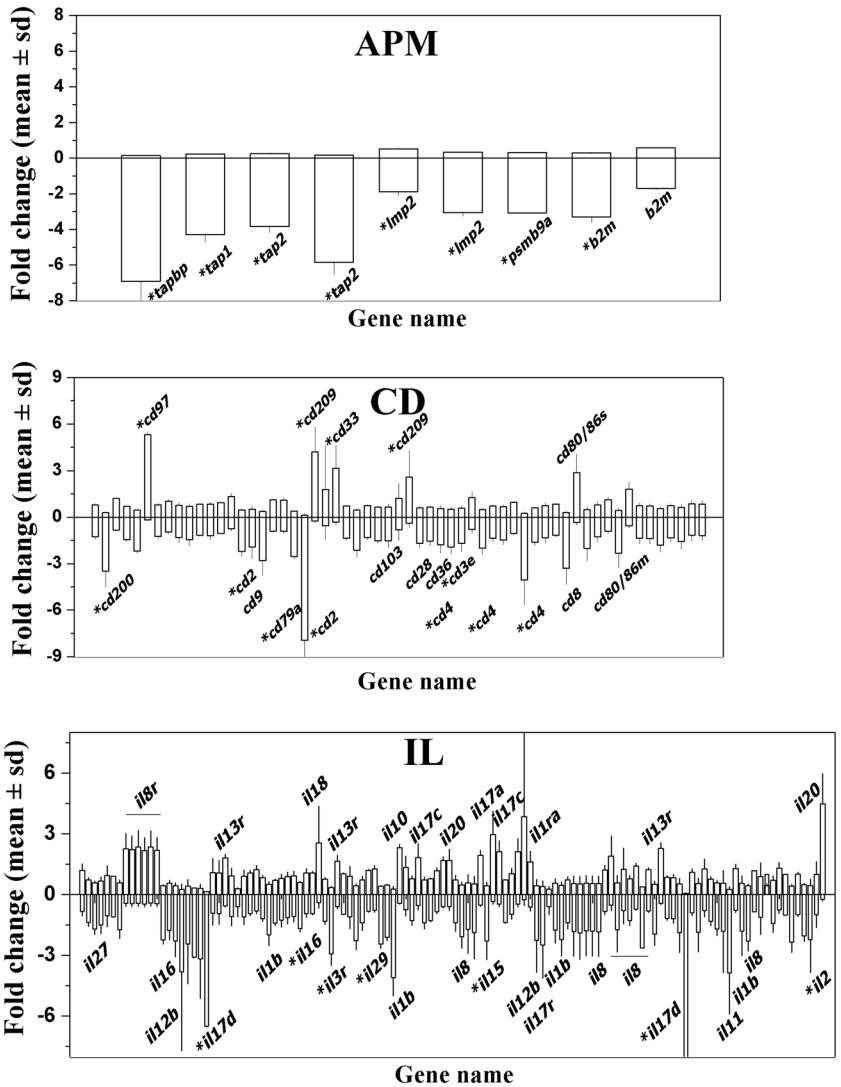

Fig. 6 Transcriptional expression changes induced by rNV among antigen presentation machinery (*APM*), cluster of differentiation (*CD*) and interleukins and their receptors (*IL*) gene groups. Differential fold changes±standard deviations are represented by *bars* (Y-axis). Probes defining distinct genes are represented in *X*-axis. *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). Fold change calculations are described in 'Materials and methods' and Fig. 4 legend. APM group, genes downregulated: *tapbp*=tapasin, TAP binding protein, *tap1*=transporter associated with antigen processing 1, *tap2*=transporter associated with antigen processing 2, *lmp2=psmb9*, *psmb9a*=proteasome subunit, type 9, *b2m*=beta-2 microglobulin. CD group, genes upregulated: *cd97*, *cd209*, *cd33*, *cd209* and *cd80/86 s* (soluble form). Genes downregulated: *cd200*, *cd2*, *cd9*, *cd79a*, *cd103*, *cd28*, *cd36*, *cd4*, *cd3e*, *cd8* and *cd80/86 m* (membrane form). IL group, *r* means receptor. Genes upregulated: *il1ra*, *il8r*, *il10*, *il13r*, *il17a*, *il17c*, *il18* and *il20*. Genes downregulated: *il2*, *il3r*, *il8*, *il11*, *il12b*, il15, *il16*, *il17d*, *il17r*, *il1b*, *il27* and *il29*

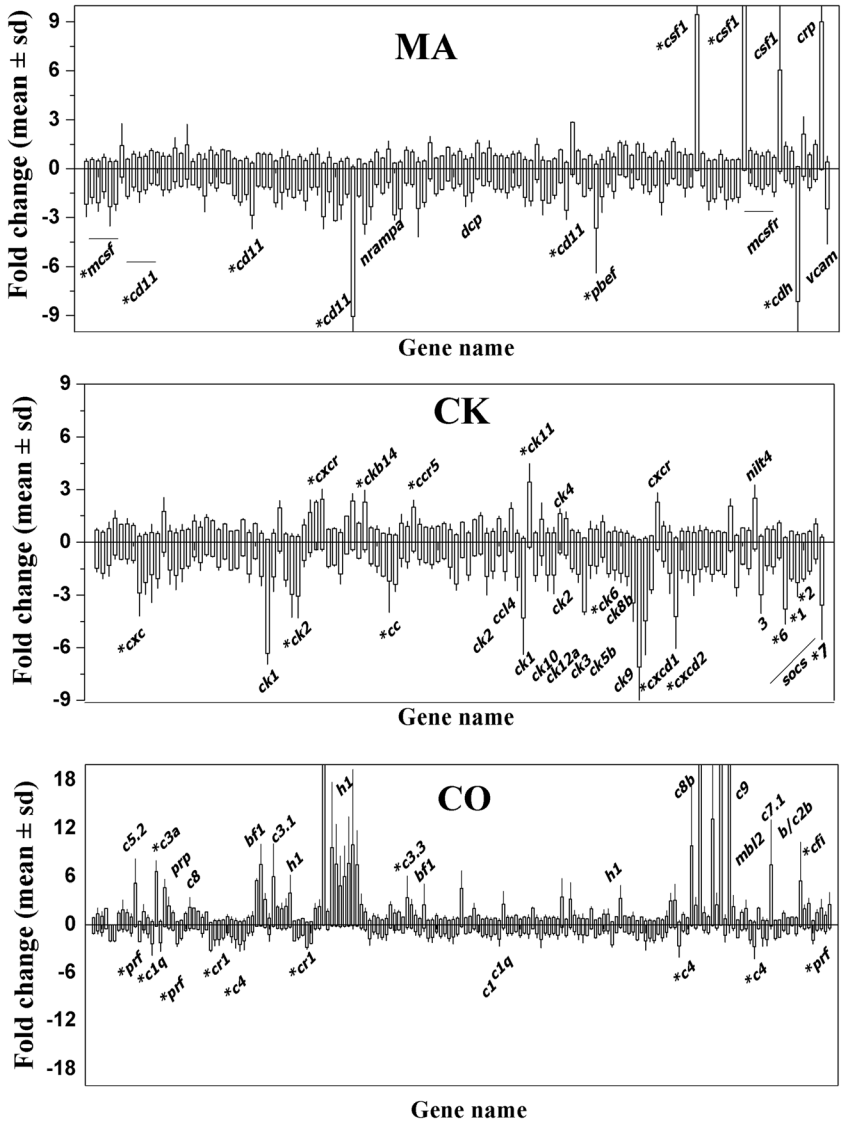

Fig. 7 Transcriptional expression changes induced by rNV among macrophage-related (*MA*), chemokines (*CK*) and complement components (*CO*) gene groups. Differential fold changes±standard deviations are represented by *bars* (*Y*-axis). Probes defining distinct genes are represented in *X*-axis. *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). Fold change calculations are described in 'Materials and methods' and Fig. 4 legend. MA group, genes upregulated: *csf1*=colony stimulating factor 1, *crp*=c-reactive protein. Genes downregulated: *mcsf*=macrophage conolony stimulating factor, *cd11*=alpha chain of many integrins, *nrampa*=natural resistance macrophage alpha, *dcp*=dendritic cell protein, *pbef*=colony pre-B enhancing factor, *mcsfr*=macrophage colony stimulating factor receptor, *cdh*=cadherin and *vcam*=vascular cell adhesion molecule-1. CK group, genes upregulated: *cxcr*=cxc receptor, *ckb14*=cc-chemokine 14, *ccr5*=cc-chemokine 5 receptor, *ck11*=cc-chemokine 11, *ck4a*=cc-chemokine 4a and *nilt4*=novel immunoglobulin-like transcript 4. Genes downregulated: *cc*=β-chemokines, *ccl4*=cc-chemokine, *ck1*=cc-chemokine 1, *ck10*=cc-chemokine 10, *ck12a*=cc-chemokine 12a, *ck2*=cc-chemokine 2, *ck3*=cc-chemokine 3, *ck5b*=cc-chemokine 5b, *ck6*=cc-chemokine 6, *ck8b*=cc-chemokine 6, *ck9*=cc-chemokine 9, *cxc*=α-chemokines, *cxcd1*=cxc d1 chemokine, *cxcd2*=cxc d2 chemokine, *socs1*=suppressor of cytokine signalling 1, *socs2*=suppressor of cytokine signalling 2, *socs3*=suppressor of cytokine signalling 3, *socs6*=suppressor of cytokine signalling 6 and *socs7*=suppressor of cytokine signalling 7. CO group, genes upregulated: *c5.2*=complement component 5, isoform 2, *c3a*=complement component 3a, *c9*=complement component 9, *prp*=properdin, *c8*=complement component 8, *bf1*=complement factor B, *c3.1*=complement component 3, isoform 1, *h1*=complement factor H1, *c3.3*=complement component 3, isoform 3, *c8b*=complement component 8b, *mbl2*=mannose-binding lectin, *c7.1*=complement component, isoform 1, *cfi*=complement factor I and *b/c2b*=complement factor B/C2b. Genes downregulated: *prf*=perforin, *c1*=component, *c1q*=component of C1 (classical complement activation pathway), *cr1*=complement receptor 1 and *c4*=complement component 4

to carry out studies of the trout transcriptome at times longer than 48 h post-injection of NV could not show real NV effects. Our main results showed that rNV induced an inhibition of multiple innate and adaptive genes. In the majority of organisms, innate immune response starts immediately upon host exposition to antigen and extends for at least 72 h, whereas the

adaptive immune response begins close to 72–96 h, later than the innate. The effect induced by rNV on transcriptome showed the downregulation (and some upregulation) of multiple genes involved in the innate and adaptive immune response, such as those participating in antigen presenting (*tap*, *lmp2*, *β2m*, *cd4*, *cd8*) and other macrophage-related genes. Some downregulated interleukins, interferons and transcription factors activated by innate immunity are involved in triggering adaptive immune responses. This could explain why the adaptive immune genes are also inhibited at 48 h post-NV treatment. The NV protein induced a loss of innate immune response and a delay of adaptive immune response, which will favour viral replication at least during the early stages of VHSV infection under a weak antiviral state. To our knowledge, this is the first report suggesting most important possible rNV targets and describing extensive transcriptional in vivo profile changes induced by the rNV protein in any fish novirhabdovirus.

Identification of *stat1* and *jun/atf1* as differentially expressed multipath genes

By using microarray data and rainbow trout immune pathways, the transcription factors *stat1* and *jun/atf1* have been identified as the downregulated multipath genes more represented in the pathways studied (>25 % of the immune pathways, Table 1). The potential of multipath genes to suggest functional studies had been used previously to investigate the effects of spring viraemia virus (SVCV) infection on zebrafish to suggest prevention of drug candidates (Encinas et al. 2013). The multipath analysis also suggested other multipath genes which were differentially expressed after rNV injection and were upstream or related to either *stat1* (i.e. interferon-related genes such as *ifng*, *ifn1*, *irf1*, *irf2*, *irf10*, *socs1*, *casp6*, *il1b*) or *jun/atf1* (i.e. tumour necrosis factor-related genes such as *tnfa*, *traf2*, *traf3*).

The mechanisms of NV action could involve the inhibition of some downregulated genes for multipath TFs found such as *stat1* (and *jun/atf1*). Our recent prelimary experiments indicated that rNV and STAT1 might interact, although further experiments need to be carried out to affirm that interaction. Signal transducer and activator of transcription (STAT1) is a cytoplasmic latent transcription factor that becomes activated by various extracellular stimuli, including IFNγ and IFN1 after binding to their specific cell surface receptors. Mammalian phosphorylated homodimers of STAT1 bind to interferon-gamma-activated sequence (GAS), while STAT1 heterodimers bind to interferon-stimulated response element (ISRE) transcription factor binding sites (TFBS). Binding to those TFBS leads to an increased expression of many *ifn*-stimulated

Table 1 List of differentially expressed genes which were present in at least six immune-related pathways ranked by the number of pathways

Short name	Accession number	Gene description	No. of paths	NV Mean	±SD	*N*
stat1	NM_001124746.1	Signal transducer/activator	27	**−4.73***	1.75	16
jun	NM_001124411.1	Jun proto-oncogen	25	**−2.94***	0.95	4
atf1	NM_001124559.1	cAMP-dependent transcription factor	20	**−1.75**	0.19	8
traf3	NM_001124615.1	Tumour necrosis factor receptor 3	14	**−2.10***	0.61	4
traf2	NM_001124393.1	Tumour necrosis factor receptor 2	13	**−1.52**	0.18	4
tnfa	AJ277604.2	Tumour necrosis factor alpha	12	**−1.45**	0.63	12
ifng	NM_001124620.1	Interferon gamma	11	**−3.11***	0.71	20
casp6	NM_001124271.1	Caspase 6	9	**−2.24***	0.89	4
irf1	NM_001124293.1	Interferon regulatory factor 1	9	**−3.80***	1.02	24
il1b	AJ223954.1	Interleukin-1-beta	8	**−4.10***	0.90	12
irf10	AJ829672.1	Interferon regulatory factor 10	8	**−2.39**	1.13	4
socs1	NM_001146166.1	Suppressor cytokine signalling 1	8	**−2.08***	0.13	4
ifn1	AM489418.1	Type I interferon 1	6	**−2.52***	0.21	28
irf2	NM_001124438.1	Interferon regulatory factor 2	6	**−1.80***	0.17	8

Differentially expressed genes with folds <0.66 (downregulated, -1/fold) present in >5 pathways (multipath genes) of the 74 screened together with their corresponding mean folds were filtered/extracted from the microarray data. The differentially expressed multipath genes were tabulated together with their corresponding mean folds (bold) and standard deviations by comparing rNV-PBS vs polyH-PBS. The genes were then ordered by the number of pathways in which they were present. Other genes common to >6 pathways were not differentially expressed, for instance, *p53* (NM_001124692.1, common to 16 pathways), *tnf6* (TC121157, 13), *il6* (NM_001124657.1, 11), *lck* (NM_001124542.1, 11), *mhc2.daa* (AJ251431.1, 10), *junb* (NM_001124520.1, 10), *if2a* (NM_001124296.1, 9), *myc* (NM_001124699.1, 9), *tgfb* (X99303.1, 9), *il12b* (AJ548830.1, 8), *il8* (AY160981.1, 7), *grb* (AY173044.1, 7) and *irak4* (FN598575.1). The *atf*, *traf2* and *tnfa* genes have been included because of their relationships to other genes in the table

"–" downregulated, *n* total number of probes per gene analysed

*Significantly different from <0.66 (<−1.5) at the $p \leq 0.05$ level

genes (*isg*) that contain those TFBS sequences in their promoters, closing the regulatory circle (IFNs-STAT1-IFNs). *Socs1* (another multipath gene) encodes a STAT1-inducible feedback inhibitor of IFN signalling. Other multipath genes downregulated by rNV, such as *il1b* or *casp6*, are also related to *stat1*. On the other hand, the products of *jun/atf1* belong to transcription factors participating in the activating protein 1 (AP-1) heterodimer which targets tumour promoter antigen (TPA) on the DNA and have many immunological effects including those related to *tnf* and *tnf*-related genes (another multipath genes downregulated after rNV injection, Table 1) (Manicassamy and Pulendran 2009; Parker et al. 2007; Schröder et al. 2006; Schröder and Bowie 2007; Takeuchi and Akira 2007; Thompson and Iwasaki 2008). The interferon system has a key role in controlling viral infections. Different kinds of viruses (RNA, DNA) have developed strategies to circumvent the IFN responses. Mammalian rhabdoviruses such as vesicular stomatitis virus (VSV) and rabies virus (RV) use the matrix protein M (M_{VSV}) and the phosphoprotein P (P_{RV}), respectively, to inhibit nuclear accumulation of phosphorylated STAT1, of binding of phosphorylated STAT1 to *isg* genes (Chelbi-Alix et al. 2006; Vidy et al. 2007). Among fish rhabdoviruses, SVCV lacks the NV gene, being NV restricted to the genus *Novirhabdovirus*, whose member VSHV has maintained the NV protein for inhibiting the host antiviral state.

Immune gene regulation induced by rNV

Microarray data were obtained from trout spleen and head kidney. Regarding the rNV delivery route, because head kidney trout cells were capable of incorporating extracellular rNV in vitro as well as in vivo (see results), intraperitoneally injected rNV might have been internalised to the cytosol of intraperitoneal macrophages and then transported to head kidney and/or spleen. Transcriptome changes might reflect either those taking place in the macrophage or those induced by proteins released from the rNV-containing macrophages in other cells in the internal organs. The possibility that the observed changes in gene expression could be due to changes in cell type abundance in head kidney and spleen due to cell migration rather than within constant cell populations (i.e. macrophages) must be also considered as a possible alternative mechanism to explain the profiles of differential expression. Future histological and/or complementary flow cytometry analysis of head kidney and spleen will be used to study likely rNV effects on cell-type abundances (ongoing work).

On the other hand, we wanted to investigate whether the precipitated rNV also had any effect on the gene expression of the immune-related genes, noting that precipitated NV had no effect (results not shown). Expression changes modulated by soluble rNV could be observed in constitutive and non-constitutive expressed genes. This might be due to manipulation and injection (PBS or rNV) of all fish leading to certain stress and subsequent upregulation of the gene expression (e.g. *mx* and *ifns*). Previous works describe that the *ifn1* gene is not constitutively expressed in rainbow trout whereas *ifn2* and *ifn3* are (Zou et al. 2007). However, other works showed a constitutive expression of many isoforms of all of them (Purcell et al. 2009). In the present work, all the trout groups injected with PBS or rNV might be a quiet initial upregulation, but only in the rNV-injected group, a downregulation is observed due to rNV effect, being relative values in the comparison rNV vs PBS.

Immune-targeted microarray whole data (spleen and head kidney) confirmed previous results on *ifn*-related and *casp* genes, and this increases the confidence in the rest of the novel data described in this work. We have characterised novel downregulated *ifn*-related genes also amongst the multipath genes (*ifn1*, *ifng*, *irf1*, *irf2*, *irf10*). The downregulation in the caspases confirmed also that rNV induces an anti-apoptotic state in the infected cells to maintain VHSV replication, as suggested by previous functional studies comparing wt- and NV knockout novirhabdoviruses (Ammayappan and Vakharia 2011).

One of the most characteristic new observations came from the VHSV-induced genes (*vigs*). These genes were previously identified by others as in vitro early responses characteristic of VHSV-infection by using subtractive suppressive hybridisation of RNAs isolated from infected head kidney rainbow trout leukocytes (O'Farrell et al. 2002). Upregulation of *vigs* was demonstrated by semiquantitative RT-qPCR and confirmed by Northern blot analysis. In vivo downregulation of most *vigs* by rNV has been described for the first time here, suggesting that one of the most significant effects of rNV is to suppress those early host responses, thus highlighting the importance of *vigs* for natural fish immunity towards novirhabdoviruses.

Similarly, most of the rainbow trout *tnf*-related (inducers of inflammatory responses) and most of the *tlr*-related genes were downregulated by rNV, indicating that rNV inhibits inflammation and detection of viral RNA such as *tlr3* (dsRNA) and *tlr7* (ssRNA). Contrary to other *tlrs*, *tlr5* is found upregulated. We cannot exclude a previous bacterial impact on trout. However, this should be reflected among all trout whatever rNV or polyH they were injected. We think that an explanation for *tlr5* upregulation might be because of the different probe binding to its complementary sequence due to a not yet described polymorphism. In this work, *tlr5* gene expression was only defined with one probe located at the 3′ end untranslated region in its mRNA. Single nucleotide polymorphisms (SNPs) within *tlr5* coding regions have been found in humans, pigs, cattle, sheep and other species (Hawn et al. 2003; Smith et al. 2012; Yang et al. 2013). However, little is known about SNPs in mRNA-untranslated regions.

Since rNV induced downregulation of antigen presentation machinery (APM) genes and since their corresponding proteins have a major role in processing antigenic proteins into peptides to charge MHC class I molecules for antigen presentation to $CD8^+$ T-cytotoxic lymphocytes, rNV could impair these host mechanisms for appropriate processing of pathogen antigens (i.e. those resulting from VHSV infection), delaying the beginning of adaptive immune response.

The cluster differentiation (*cd*) genes downregulated by rNV included the most characteristic T-helper and T-cytotoxic *cd4* and *cd8* markers, respectively, suggesting also downregulation of the beginning of adaptive immune responses. Another example of adaptive immune signalling interference by rNV was downregulation of *cd28*, similarly to what has been reported in mammalians with the NEF protein of HIV-1 (El-Far et al. 2013). Other data on *cds* might indicate that rNV also stimulates immune responses like any other protein antigen, which could have been injected, for instance, downregulation of *cd200* (NK immunosuppression) and upregulation of *cd97*, *cd209*, *cd33*, etc.

There was an extensive interleukin (*il*) upregulation and downregulation induced by rNV which might have extensive implications for the immune response. Thus, downregulation of *il1b* and *il8* could cause downregulation of *nfkb* and therefore hamper the immune responses in many other pathways. Regarding *il17*, while *il17a* and *il17*c were upregulated by rNV, *il17d* (affecting *il1b* and *tnfa*) was downregulated by rNV. The transcripts of *Il17a* are produced in multiple cell types including CD4+αβ, γδ T-cells, natural killers and neutrophils, and IL17a protein is an inducer of granulocyte/monocyte stimulating factor (*gcsf*), several chemokines and neutrophil recruitment factors (Kolls and Linden 2004). In mouse, the *il17a* gene was upregulated by *il23* in *soc3* (activation of macrophages)-deficient mice (Aggarwal et al. 2003). In those conditions, IL23 induced STAT3 phosphorylation, which in turn increased *il17a* gene transcription. Therefore, *il17a* upregulation in trout may be caused by downregulation of *soc3* by rNV.

Downregulation of *nramp*, *pbef* and *cd11* by rNV might have important consequences for the responses of macrophages to VHSV infection and/or to the carry-over of NV by macrophages. The *nramp* gene family codes for macrophage membrane proteins related to phagocytosis (Govoni and Gros 1998), while the protein encoded by *pbef* is an inhibitor of apoptosis (by inhibiting CASP3 and CASP8) (Jia et al. 2004) and *cd11* encodes for an important dendritic cell marker. Therefore, downregulation/upregulation of these genes by rNV suggests their effects on inhibition of phagocytosis, infection-defensive apoptosis and antigen processing and presentation.

Some chemokine (*ck*) gene modulations were similar to those previously reported in trout fins after bath infection with VHSV (i.e. upregulation of *ck11* and downregulation of *ck10* or *ck12*) (Montero et al. 2011) or epidermis (*ck6* and *ck9*). The rNV upregulation of *inos* might favour VHSV replication since increase in nitric oxide (NO) described in turbot macrophages infected with VHSV showed that NO had no antiviral effects (Tafalla et al. 1999). Other differential expression of cytokines remains to be explained. There are no reported studies with both *socs1* (involved in T_h and T_{reg} regulation) (Lu et al. 2009; Yoshimura et al. 2012) and *socs3* (Liu et al. 2008) being downregulated by any other viral protein.

Antibody recognition at the beginning of the classical complement pathway seems to be inhibited by rNV by downregulation of *c1q* similarly to *prf* (perforin) implicated at the end in membrane pore formation. In contrast, *h1* (an inhibitor of complement) and most genes implicated in the final attack complex (*c5*, *c7*, *c8*, *c9*) were upregulated. These results suggest that rNV modulates complement components to delay any possible antibody and/or cellular-mediated responses and that host responses try to maintain some complement components activated. This scenario might reflect the host/pathogen fight at the beginning of VHSV infection.

In vivo protection had been reported for VHSV challenge with NV knockout VHSV in flounder (Kim et al. 2011) and for IHNV challenge with NV knockout IHNV for trout (Thoulouze et al. 2004) as live-attenuated vaccine candidates in a dose-dependent manner (Kim et al. 2011; Thoulouze et al. 2004). Data presented in this work could explain much better than before the protection induced by knockout novirhabdoviruses as due to the lack of an extensive inhibition of host early immune gene responses. Forthcoming studies will be focused on the characterisation of molecular targets for NV (e.g. *stat1*, *atf1/jun*, *nfκβ*). The results described in this work, novel in the use of rNV in vitro/in vivo and of immune-targeted microarrays, produced a better understanding of additional possible NV functions and suggested some working hypothesis to continue the work on NV effects. The novel data obtained improves our knowledge of the immunosuppression mechanisms used by VHSV, and similar strategies could be used with other novirhabdoviruses. The results of this work also open up the possibility to use rNVs as a new tool to further study immune response mechanisms in other teleost fish.

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