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Transcriptome profiling of the antiviral immune response in Atlantic cod macrophages

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Abstract

A study was conducted to determine the transcriptome response of Atlantic cod (*Gadus morhua*) macrophages to the viral mimic, polyriboinosinic polyribocytidylic acid (pIC), using a 20K Atlantic cod microarray platform and qPCR. We identified 285 significantly up-regulated and 161 significantly down-regulated probes in cod macrophages 24 h after pIC stimulation. A subset of 26 microarray-identified transcripts was subjected to qPCR validation using samples treated with pIC or phosphate-buffered saline (control) over time (3, 6, 12, 24, 48 h), and 77% of them showed a significant response to pIC. The microarray and qPCR analyses in this study showed that pIC induced the expression of cod macrophage transcripts involved in TLR-dependent pathogen recognition (e.g. *tlr3, tlr7, mda5* and *lgp2*), as well as signal transducers (e.g. *stat1* and *nfkbia*) and transcription activators (e.g. *irf7* and *irf10*) in the MyD88-independent and dependent signalling pathways. Several immune effectors (e.g. *isg15*s, *viperin*, *herc4*, *mip2* and *ccl13*) were significantly up-regulated in pIC-stimulated cod macrophages. The expression of some transcripts (e.g. *irf7*, *irf10*, *viperin*) was significantly up-regulated by pIC as early as 12 h. All pIC-induced transcripts had peak expression at either 24 h (e.g. *tlr7, irf7*, *mip2*) or 48 h (e.g. *tlr3*, *lgp2, stat1*). This study suggests possible roles of both vertebrate-conserved (e.g. *tlr3* as an up-regulated gene) and fish-specific (*tlr22g* as a down-regulated gene) receptors in dsRNA recognition, and the importance of conserved and potentially fish-specific interferon stimulated genes in cod macrophages.

Key words: microarray, qPCR, poly(I:C), fish, pathogen recognition, *Gadus morhua*

Running title: Atlantic cod macrophage antiviral gene expression response

1. Introduction

In addition to the importance of the Atlantic cod (*Gadus morhua*) fishery for several countries (e.g. Canada, Norway and Finland), this species is regarded as a promising candidate for northern Atlantic aquaculture (Rosenlund and Halldórsson, 2007). Atlantic cod aquaculture has been developing in several countries (e.g. Canada, Norway and Iceland) as a result of the over-exploitation and dramatic decline of some wild cod populations (Johansen et al., 2009). The development of large-scale Atlantic cod aquaculture faces serious issues including viral diseases [e.g. viral nervous necrosis (VNN), caused by nodaviruses] (Samuelsen et al., 2006). Identification of the transcripts and proteins involved in antiviral immune responses of Atlantic cod [e.g. pattern-recognition receptors (PRRs), transcription activators and immune effectors] can improve the health management of wild and farmed fish, e.g. through development and application of molecular biomarkers for assessing virus carrier state and response to vaccines or therapeutants. Further, since the Atlantic cod possesses a unique immune system among bony fishes [e.g. high serum IgM concentration, weak specific antibody responses, a large number of MHC I (major histocompatibility complex I) paralogues and the absence of transcripts encoding MHC II and CD4 proteins] (Solem and Stenvik, 2006; Star et al., 2011), understanding the genes and mechanisms involved in immune responses in this species is of evolutionary importance.

Polyriboinosinic polyribocytidylic acid (pIC) is a synthetic double-stranded RNA (dsRNA) viral mimic that can trigger antiviral responses [e.g. expression of IFNs (interferons) and ISGs (IFN-stimulated genes)] in fish as effectively as pathogenic viruses and more efficiently than other pathogen-associated molecular patterns (PAMPs) such as CpG oligodeoxynucleotides (Strandskog et al., 2008). Nodaviruses are thought to produce dsRNA intermediates in their replication cycle (Wu et al., 1992), and pIC was shown to increase the

resistance of grouper fish (*Epinephelus septemfasciatus*) against VNN challenge (Nishizawa et al., 2011). pIC, mainly recognized by various PRRs in fish [e.g. TLRs (Toll-like receptors)], activates the signalling pathways that produce IFNs. Correspondingly, the binding of IFNs to IFNRs (IFN receptors) leads to the transcription of ISGs through activation of the JAK-STAT signalling pathway (reviewed by Langevin et al., 2013b; Zhang and Gui, 2012).

Microarray analysis of gene expression can provide a profile of transcriptional responses of cells or tissues to various agents (e.g. bacteria and viruses) (Hyatt et al., 2006). Using a database containing over 150,000 expressed sequence tags (ESTs) identified in various suppression subtractive hybridization (SSH) and normalized cDNA libraries (including several libraries designed to be enriched with virus-responsive transcripts), a 20,000 probe (20K) custom-built oligonucleotide (oligo) Atlantic cod microarray platform was previously designed and quality tested (Booman et al., 2011). This 20K microarray platform has been used to study cod oocyte and larval transcriptomes (Rise et al., 2015; Rise et al., 2014), as well as the effects of diet or elevated temperature on the cod spleen transcriptome responses to immunogenic stimuli (e.g. pIC) (Booman et al., 2014; Hori et al., 2012; Hori et al., 2013). The current study is the first application of the 20K cod microarray platform to study the macrophage transcriptome.

PRRs that initiate antiviral immune responses, and consequently activate immune signalling pathways, could be tissue- or cell-specific in a species (Langevin et al., 2013a). For example, a tissue-dependent response to viral challenge was reported for teleost-specific *tlr*s (e.g. *tlr22*) of Atlantic cod (Sundaram et al., 2012). Macrophages play prominent roles in fish immune responses on the cellular and molecular levels through pathogen recognition (e.g. different PAMPs), regulation of innate and adaptive immune responses, cytokine production [e.g. TNFs (tumour necrosis factors) and ILs (interleukins)], antigen presentation, and

phagocytosis (Roca et al., 2007; Whyte, 2007). Viral infections increase IFNs and nitric oxide production, phagocytosis, and respiratory burst activity in fish macrophages (Collet, 2014). Viral infections also alter host gene expression, for example microarray analyses showed a massive transcriptomic response of Atlantic salmon macrophage-like cells to infectious salmon anaemia virus, ISAV (Schiøtz et al., 2008; Workenhe et al., 2009). Bakkemo et al. (2011) showed the time-dependent induction of ILs-encoding transcript expression by lipopolysaccharide (LPS) in Atlantic cod macrophages. Using real-time quantitative polymerase chain reaction (qPCR) and a small microarray platform (1056 probes), only 4 pIC-responsive transcripts (i.e. *isg15*, *bty*, *tlr3* and *enolase 1 α*) were identified in Atlantic cod head kidney cells (Holen et al., 2012). However, a larger microarray platform that provides better coverage of the transcriptome (as used in the current study) will yield a more complete picture of the antiviral response of head kidney cells or a specific type of Atlantic cod leukocyte (e.g. macrophages). Therefore, the present study aimed to determine the transcriptome profile of the antiviral response of Atlantic cod macrophages, using the 20K Atlantic cod microarray and qPCR.

2. Materials and methods

2.1. Animals

Atlantic cod (1.09 ± 0.73 kg), reared in the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre (OSC), were used for this experiment. The fish were kept in a 21 m^3 tank and optimum conditions (5.2-6.4˚C, 95-110% oxygen saturation and under an ambient photoperiod). The fish were fed 3 days per week at a level of 1% body weight per feeding time, using a commercial diet (Skretting, BC, Canada; crude protein 50%, crude fat 18%,

crude fibre 1.5%, calcium 3% and phosphorus 1.4%). All procedures in this experiment were approved by Memorial University of Newfoundland's Institutional Animal Care Committee, based on the guidelines of the Canadian Council on Animal Care.

2.2. Macrophage isolation

Atlantic cod head kidney macrophages were isolated based on Bakkemo et al. (2011) and Steiro et al. (1998) with some modifications. Briefly, the fish were netted and immediately euthanized with an overdose of MS222 (400 mg L^{-1}; Syndel Laboratories, Vancouver, BC, Canada); blood was removed from the caudal vein of each fish using a 5 ml syringe with a 25 gauge needle. Each fish was then dissected, and the hematopoietic kidney (i.e. head kidney) was removed and minced through 100 µm nylon cell strainers (Fisherbrand™, Thermo Fisher Scientific, Waltham, MA, USA) in Lebovitz-15+ (L-15+; Gibco, Carlsbad, CA, USA) culture medium supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 25 mM HEPES, 1.8 mM glucose, 20 U ml^{-1} heparin (Sigma-Aldrich, St. Louis, MO, USA) and 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Gibco). The cell suspension was centrifuged on a discontinuous 25/51% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 300 × g for 40 min at 4˚C, and the macrophage-enriched interface was collected. The resulting macrophage-rich suspension was washed twice in L-15+ by centrifuging at 300 × g for 15 min at 4 ˚C. The cells were diluted in L-15+ medium containing 1% fetal bovine serum (FBS; Gibco) and without heparin in the last washing stage. The viable cells were counted using a hemocytometer and a trypan blue (Sigma-Aldrich) exclusion test, and above 96% viability was recorded for isolated cells from all fish. Thereafter, the cells were seeded into 35 mm culture dishes (Corning, Corning, NY, USA) at an equal density of 3×10^7 cells (in 2 ml L-15+) per dish. The cells were allowed to adhere overnight (16 h) at 10˚C, and then the non-adherent cells were removed by washing the culture

dishes 3 times with L-15+. The adherent, macrophage-enriched cells are henceforth referred to as macrophages (see section 2.3).

2.3. Cell characterisation

To determine the effect of pIC on cell death, size and complexity, macrophages were isolated from 3 Atlantic cod and cultured in 6-well plates (Corning, Corning, NY, USA) as in section 2.2. Samples were plated in duplicate wells. After 24 h in culture, cells were stimulated with 50 µg ml^{-1} pIC (as determined in section 2.4) or PBS (control) for 24 and 48 h. Cells were removed from the plate using trypsin-EDTA (0.25%) (Thermo Fisher Scientific, Waltham, MA, USA), washed in 1 ml of fluorescence-activated cell sorting (FACS) buffer (PBS + 1% FBS) for 5 min, 500 × *g*, at 4ºC, and stained with 1.0 µg ml^{-1} propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA) (Kalgraff et al., 2011). Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analysed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). Cell death was determined as PI positive events, while changes in morphology were determine via visual assessment of changes in cell size (forward scatter, FSC) and cell complexity (side scatter, SSC). To determine the effect of pIC and sampling time on cell differentiation, cells from the pIC and control groups were stained using Wright-Giemsa stain (Thermo Scientific) at 24 and 48 HPS, according to the manufacturer's instructions. The percentage of macrophage-like cells [i.e. large, adherent cells with cytoplasmic projections (Sørensen et al., 1997)] was determined in three fields of view (magnification 200X) for each sample.

2.4. Macrophage stimulation

Six individual Atlantic cod were used for determination of the most stimulative dose of pIC and microarray-based identification of antiviral (i.e. pIC-responsive) macrophage transcripts (see Supplemental Figure S1). For each individual cod, macrophages were isolated and seeded into five 35 mm culture dishes (i.e. a total of 30 dishes from 6 fish). The viral mimic pIC (Sigma-Aldrich) was dissolved in sterile phosphate-buffered saline (PBS) (pH: 7.2). Starting 24 h after isolation, the macrophages from each individual were exposed to a range of pIC doses including 0 [PBS (control)], 1, 10, 50, and 100 µg ml^{-1} at 10˚C. A previously published study showed that the highest transcript expression of Atlantic cod *isg15* in response to pIC occurred 24 h post-stimulation (HPS) (Seppola et al., 2007). Therefore, 24 h after the onset of stimulation (i.e. 48 h after isolation), the media was removed and macrophages were harvested by adding 800 µl TRIzol (Invitrogen, Burlington, Ontario, Canada) into each culture dish. Samples were kept at -80˚C until RNA extraction. qPCR for known antiviral biomarker transcripts (*isg15*, *viperin* and *stat1*, see sections 2.5 and 2.8.2) was used to determine that 50 µg ml^{-1} pIC was the optimal dose of pIC (data not shown). Therefore, this treatment group was selected for microarray analysis.

2.5. RNA extraction and purification

Total RNA was extracted from TRIzol-lysed samples following the manufacturer's instructions, and RNA pellets were dissolved in 50 µl DNase/RNase-free water (Gibco). The RNA samples were treated with DNase-I (Qiagen, Mississauga, Ontario, Canada) to degrade residual genomic DNA, and then purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were measured using NanoDrop spectrophotometry (ND-1000), and the RNA integrity was checked by agarose gel electrophoresis (1% agarose gel). The high-purity RNA samples (e.g. A260/230 and

A260/280 ratios > 1.8) with tight 18S and 28S ribosomal RNA bands were used for the gene expression analyses (i.e. microarray and qPCR).

2.6. Microarray experimental design and hybridization

This microarray experiment was designed and carried out based on the MIAME guidelines (Brazma et al., 2001). Macrophages exposed to 50 µg ml^{-1} pIC or PBS (control) for 24 h (pIC and PBS treatments for each of 6 individuals) were used to identify the pIC-responsive transcripts in Atlantic cod macrophages (See Supplemental Figure S1). The Atlantic cod 20K oligonucleotide microarray platform (Booman et al., 2011) was used for this reference design experiment that included 12 arrays. One microgram of DNase-treated and column-purified RNA was amplified and then labelled using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, Carlsbad, CA, USA), according to the manufacturer's protocol. The amplified RNA (aRNA) samples were column-purified and their quality and quantity assessed using gel electrophoresis and NanoDrop spectrophotometry, respectively. A common reference was prepared using a pool of all 12 experimental samples (15 µg aRNA from each sample). The individual experimental samples (pIC and control) were labelled with Cy5 (GE Healthcare Life Sciences, Buckinghamshire, UK), whereas the common reference was labelled with Cy3 (GE Healthcare Life Sciences), based on the manufacturer's protocol (in the aRNA Amplification kit). Then, 3 µg of labelled aRNA was fragmented using Ambion Fragmentation Reagents (Ambion). For each array, 3 µg of labelled aRNA of one experimental sample, 3 µg of labelled aRNA of the common reference and 2 µl of LNA blocker (Genisphere, Hatfield, Pennsylvania, USA) were mixed in a formamide-based hybridization buffer (Genisphere) and hybridized to the array overnight (~ 16 h) at 42˚C (Booman et al., 2011). The detailed protocols for hybridizations and washing are described in Booman et al. (2011).

2.7. Microarray data acquisition and analyses

The arrays were scanned at 5 µm resolution using a ScanArray Gx Plus scanner and ScanExpress v4.0 software (Perkin Elmer, Waltham, Massachusetts, USA). Photomultiplier tube (PMT) settings were adjusted for Cy5 and Cy3 to minimize overall signal differences between channels. The raw data were saved as Tiff images, and then Imagene 9.0 (BioDiscovery, El Segundo, California, USA) was used for extracting the signal intensity data. In R and the Bioconductor package marray, the flagged spots were omitted and the resulting data were $\log_2$-transformed and Loess-normalized per subgrid (Booman et al., 2014). Probes with more than 25% missing values in all 12 arrays were removed from the data file, and the missing data were imputed by means of the EM_array method, using the LSimpute package (Bø et al., 2004; Celton et al., 2010). Thereafter, the final data file was composed of 11,797 probes for all arrays (GEO accession number: GSE70808).

The significant differences in transcript expression were found using Significance Analysis of Microarrays (SAM) (Chu et al., 2001; Tusher et al., 2001), as implemented by the Excel Add-in SAM package (Stanford University, CA, USA). A two-class paired comparison analysis with a false discovery rate (FDR) cutoff of 0.05 (i.e. 5%) was performed using SAM to determine the differentially expressed probes between pIC and control groups. The significant transcript list was re-annotated by BLASTx aligning the differentially expressed cod sequences (i.e. contigs or singletons that were used for 20K Atlantic cod microarray design) against NCBI's non-redundant (nr) amino acid sequence database (E-value < 1.00E-05), using Blast2GO software (BioBam Bioinformatics S.L., Valencia, Spain) (Conesa et al., 2005; Götz et al., 2008). Blast2GO was also used for mapping the significant BLASTx hits to Gene Ontology (GO) terms and creating the GO pie chart of pIC-responsive transcripts (GO Biological Process level 2). GO

enrichment analysis was conducted using Fisher's Exact test (FDR cutoff of 0.05) in Blast2GO software. A bar chart of enriched GO terms, reduced to the most specific GO terms (Biological Process and Molecular Function), was created. Selected pIC-responsive transcripts that had the same BLASTx hit name were re-assembled using the SeqMan function in Lasergene 7.20 software package (Madison, WI, USA) to determine if those transcripts represented different putative paralogues. Finally, the median-centred data of differentially expressed probes were hierarchically clustered using the Pearson correlation and complete linkage clustering function in Genesis software (Rockville, Maryland, USA) as in Booman et al. (2014).

2.8. qPCR validation

2.8.1. Macrophage isolation and time point sampling

To validate the microarray results, and to study the pIC response of selected microarray-identified transcripts over time, macrophages were isolated from 6 additional healthy appearing Atlantic cod and seeded in 35 mm culture dishes (10 dishes per fish, for a total of 60 dishes) as previously described in section 2.2. Macrophages isolated from each individual were exposed to either 50 µg ml^{-1} pIC or PBS starting at 24 h post-isolation. The macrophages from each individual were incorporated into both experimental groups and all time points (i.e. 6 biological replicates for each treatment and time point; see Supplemental Figure S1). The stimulated and non-stimulated macrophages were lysed as previously described at 3, 6, 12, 24 and 48 HPS. Each RNA sample was extracted, DNase-treated, column-purified, and subsequently quantity- and quality-checked as described previously.

2.8.2. qPCR analysis

cDNA was synthesized for each sample using 800 ng of RNA, random hexamer primers (250 ng; Invitrogen), 1 µl of dNTPs (10 mM each), 4 µl of 5X first-strand buffer and M-MLV reverse transcriptase (200U; Invitrogen) at 37˚C for 50 min as recommended in the manufacturer's instructions.

This qPCR experiment was conducted according to the MIQE guidelines (Bustin et al., 2009). Viral mimic-responsive transcripts representing various biological functions (e.g. pathogen recognition, signal transduction/transcription control, and immune effectors) and different response patterns (e.g. high fold-change versus low fold-change; up-regulated versus down-regulated transcripts) were selected for qPCR validation with macrophage samples from all 5 time points (Table 1). We also added 6 important immune-related transcripts (*ifng*, *ifngr1*, *p67-phox*, *bcl-x1*,*il8* and *ilb1*) that were not present in the microarray significant transcript list to the qPCR experiment. The candidate normalizer transcripts were selected from the microarray data (i.e. transcripts that were comparably expressed between pIC and PBS control groups) or from previous studies (Booman et al., 2014; Inkpen et al., 2015). Primers for the transcripts of interest and candidate normalizers were either designed using Primer3web v4.0.0 (http://primer3.wi.mit.edu) or obtained from previous studies (Booman et al., 2014; Hori et al., 2012; Inkpen et al., 2015) (Table 1). The quality and amplification efficiencies of all primer pairs were assessed using two templates (a pool of 3 individuals in the pIC group at 24 HPS for up-regulated transcripts, and a pool of 3 individuals in the PBS control group at 24 HPS for down-regulated transcripts) with technical duplicates of a 5-point (or 4-point for *tlr7*, *znfx1*, *traip*, *bcl-x1*, *mig1*, *herc4* and *catalase*), 3-fold serial dilution of cDNA, starting at 10 ng input RNA. The primer sets having 80-100% amplification efficiency (Pfaffl 2001), no amplification in the no-template controls, and a single melting peak (i.e. no evidence of primer dimers in the dissociation

curve) were selected for qPCR assays. The absence of genomic DNA contamination in RNA samples was checked with all of the selected primer sets, using no-reverse transcription controls (i.e. samples devoid of reverse transcriptase enzyme during cDNA synthesis) from pooled control and pIC samples of each fish.

To determine the optimal normalizers, the expression of candidate normalizers [*cypa* (*cyclophilin a*), *rplp1* (*60S acidic ribosomal protein P1*), *rpl4a* (*60s ribosomal protein l4-a*), *tubb2* (*beta-2 tubulin*), *hsc70* (*heat shock cognate 70 kDa*), *eif3* (*eukaryotic translation initiation factor 3 subunit*), *eif4* (*eukaryotic translation initiation factor 4*), and *eef1a* (*eukaryotic elongation factor 1 α*)] was assayed in triplicate for one-half of the samples. Using the geNorm analysis in the qBase software, two functionally distinct normalizers (*eif3* and *rplp1*) that were expressed at a stable level (i.e. with low M-value, a measure of transcript expression stability) across all samples were selected for qPCR assays as in Xue et al., (2015). The qPCR assays of the transcripts of interest were performed in 384 well plates using Power SYBR Green Master Mix (Applied Biosystems, Burlington, Ontario, Canada) and a ViiA7 Real-Time PCR system (Applied Biosystems). The expression of each transcript was measured in triplicate in a total reaction volume of 13 µl consisting of 4 µl cDNA input (corresponding to 4 ng of input total RNA for each reaction), 6.5 µl Power SYBR Green Master Mix, 1.04 µl primers (forward and reverse primers 1.25 mM) and 1.46 µl nuclease-free water (Gibco). The qPCR program was comprised of one cycle of 50 ˚C for 2 min, one cycle of 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min, with fluorescence data detected at the end of each cycle. The robustness of assay performance between plates was checked using an inter-plate linker sample [C_T (threshold cycle) value variations < 0.5] as well as no-template controls. ViiA7 software v1.2.2 (Applied Biosystems) was applied to acquire the C_T values, and also to calculate the

relative quantity (RQ) values. RQ values were calculated relative to a baseline sample (calibrator, i.e. with the lowest expression) for each transcript of interest using the amplification efficiency of each primer pair (Pfaffl, 2001).

2.8.3. qPCR data analyses

RQ values were used for statistical analyses. The normality of data was initially checked with the Kolmogorov-Smirnov normality test. The gene expression data were analysed using a repeated measures two-way ANOVA test, followed by Sidak multiple comparisons *post hoc* test in order to identify significant differences between pIC and PBS control groups at each time point, and within one group in multiple time points. Differences were considered statistically significant when $p \leq 0.05$. All data analyses were performed using Prism package v6.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Cell characterisation

PI staining remained under 4% during the culture period. The cell death slightly increased in both groups at 48 HPS (control, 2.03 ± 0.14% and pIC, 2.35 ± 0.36%) compared to 24 HPS (control, 1.08 ± 0.19% and pIC, 1.68 ± 0.44%). However, cell death was not influenced by pIC stimulation. Based on visual assessment of the flow cytometry dot plots, there was no apparent change in cell size (FSC), or cell complexity (SSC), due to culture time or pIC treatment (Supplemental Figure S2). The majority of the adherent cells were identified morphologically as macrophage-like cells; the percentage of macrophage-like cells was not affected by sampling

time or pIC at 24 HPS (control, 78.72 ± 2.25% and pIC, 75.86 ± 1.61%) or 48 HPS (control, 76.47 ± 1.24% and pIC, 72.48 ± 0.51%). Supplemental Figure S3 illustrates an example of cultured adherent cells.

3.2. The pIC-responsive transcripts in Atlantic cod macrophages

A two-class paired comparison of SAM analysis (FDR < 0.05) was used to identify the pIC-responsive transcripts in Atlantic cod macrophages at 24 HPS. We found 446 pIC-responsive probes (285 significantly up-regulated and 161 significantly down-regulated probes). After re-annotating the significant transcript list, 92 up-regulated and 99 down-regulated probes remained unknown (see Supplemental Table S1 for the complete list of pIC-responsive transcripts). A selection of pIC-responsive transcripts with putative roles in immune responses and/or macrophage functions is represented in Table 2. Some immune effector transcripts (e.g. *mip2*, *isg15*, *mig1* and *viperin*) were found to be highly responsive to pIC (more than 6-fold increase in expression). We also observed highly responsive (*lgp2*, 5.7-fold) and less responsive (*mda5*, *tlr3* and *tlr7*, 1.8 to 2-fold) up-regulated and down-regulated (*tlr22g*, -1.27-fold) transcripts involved in pathogen recognition. Transcripts having roles in signal transduction/transcription control were identified as being up-regulated (e.g. *stat1*, *irf7*, *irf10*, *nfkbia*, 1.6 to 3.1-fold) or down-regulated (e.g. *ccr7*, -1.47-fold) in response to pIC.

3.3. GO terms and GO enrichment analyses of pIC-responsive transcripts

GO terms (Molecular Function, Biological Process, or Cellular Component categories) of pIC-responsive transcripts were mapped using Blast2GO (see Supplemental Table S1). The GO annotation distribution (Biological Process level 2) of up- and down-regulated transcripts is illustrated in Fig. 1A and B. The proportion of up-regulated transcripts with GO annotation

involved in immune system process and multi-organism process appeared to be higher than that of down-regulated genes. GO enrichment analysis was performed to identify the over- and under-represented GO terms of the pIC-responsive probes compared to the whole array. We identified 315 significantly enriched GO terms (see Supplemental Table S2). After reduction to the most specific terms, 64 significantly enriched GO terms (Molecular Function and Biological Process categories) remained and these are depicted in Fig. 2. The majority of enriched GO terms were over-represented, and mainly associated with immune response signalling pathways (e.g. negative/positive regulation of MDA5, RIG-I and TLR signalling pathways) and immune effector production (e.g. negative/positive regulation of IFN and IL production). The GO terms 'oxidation-reduction process' and 'oxidoreductase activity' were significantly under-represented among pIC-responsive transcripts (see Supplemental Table S1).

3.4. Hierarchical clustering analysis of microarray results

We performed hierarchical clustering analysis to determine if different groups (pIC and control) were separated based on their transcriptome profiles. All samples were clustered using the median-centred values of 446 differentially expressed probes (Fig. 3A). We also hierarchically clustered all samples based on a subset of pIC-responsive transcripts that were annotated with GO terms associated with TLR and RLR signalling pathways (Fig. 3B). As expected, the samples clustered in two groups according to treatment (pIC or control); however, the level of immune responsiveness of macrophages to pIC differed between individuals.

3.5. qPCR validation

To validate the microarray results and to assess the time-dependent expression of pIC-responsive transcripts, Atlantic cod macrophages were exposed to pIC and sampled at different

time points. We selected a subset of pIC-responsive transcripts (i.e. 26 microarray-identified and 6 other important immune-responsive transcripts) that were related to various immunological pathways for qPCR validation. All of the microarray-identified transcripts subjected to qPCR were validated as pIC-responsive, except for *traip*, *ccr7*, *grp94* and *vimentin*. No significant change was found between pIC and control groups for any of the assayed transcripts at 3 and 6 HPS.

The qPCR results of transcripts involved in pathogen recognition are shown in Fig. 4. The highest expression of *tlr3*, *lgp2* and *mda5* in response to pIC was observed at 48 HPS, while the expression of *tlr7* in the pIC group peaked at 24 HPS. The expression of *tlr22g* transcript decreased in the pIC group at 24 HPS compared with the time-matched controls; there was, however, a significant down-regulation of this transcript in both pIC and control groups at 48 HPS compared to the other time points (Fig. 4C). The maximum induction of *lgp2* in response to pIC was approximately one order of magnitude higher (~ 34-fold) than that of other pathogen recognition-associated transcripts, i.e., *tlr3*, *tlr7* and *mda5* (3.8 to 4.6-fold).

The qPCR results of pIC-responsive transcripts involved in signal transduction/transcription control are shown in Fig. 5. *stat1* and *znfx1* were significantly up-regulated by pIC only at 24 and 48 HPS, compared to both time-matched controls and other sampling times within the pIC group. Both *irf7* and *irf10* were significantly up-regulated at 12, 24, and 48 HPS, with maximum *irf7* induction at 24 HPS (10.6-fold) and maximum *irf10* induction at 48 HPS (12.8-fold); *irf7* transcript expression in pIC-stimulated macrophages at 48 HPS was significantly lower than in pIC-stimulated macrophages at 12 and 24 HPS, although still significantly higher than the time-matched control (Fig. 5C,D). The microarray results were validated for *nfkbia*, a slightly up-regulated pIC-responsive transcript (1.3-fold at 48 HPS). The

expression of *traip*, *ccr7* and *ifngr1* did not significantly change in response to pIC, yet their expression varied in either both groups (i.e. *traip* and *ccr7*) or only the pIC group (*ifngr1*) over time. The expression of *bcl-x1* did not change in Atlantic cod macrophages in response to either sampling time or pIC stimulation (Fig. 5I).

The qPCR results of pIC-responsive transcripts with well-known or putative roles as immune effectors are depicted in Figs. 6 and 7, respectively. All three paralogues of *isg15* were strongly up-regulated by pIC, and their fold-change increased over time in the pIC group, peaking at 48 HPS. *isg15-3* was found to be more responsive to pIC (296.2-fold increase at 48 HPS) when compared to 2 other paralogues of *isg15*, but the level of responsiveness of this transcript was highly variable. Out of all *isg15* paralogues, *isg15-2* showed the lowest fold-change (117.5-fold at 48 HPS) and biological variability in the response to pIC. Likewise, *viperin*, *herc4* and *mig1* were up-regulated by pIC in a time-dependent manner, and the expression of these transcripts significantly increased within the pIC group after the 12 h time point, peaking at 48 HPS (i.e. 79.4- 38.7- and 12.9-fold respectively; Fig. 6E, 7E and 7B). Furthermore, *ifng* and *ccl13* showed a strong up-regulation in response to pIC (Fig. 6D and 6F); but, in contrast to *viperin* or *isg15*s, their expression within the pIC group did not significantly increase after the 24 h stimulation time point. Similar to the microarray results, *mip2* had the highest fold-change in response to pIC (318.2-fold at 24 HPS and 644.7-fold at 48 HPS). Two studied paralogues of *bty* had variable responses to pIC. *Bty-1* was significantly up-regulated in a time-related manner 12-48 h after pIC stimulation, with the highest expression at 48 HPS; however, the expression of *bty-2* was not induced by pIC. The expression of *il8* was not markedly induced by pIC, albeit this transcript was significantly up-regulated within both groups at 24 and 48 HPS in comparison with other sampling times. A similar time-dependent up-

regulation was observed for *il1b* in both groups. On the other hand, the expression of of *p67-phox* was not affected by pIC or sampling time (Fig. 6I). Similar to our microarray result, *ctsa* was down-regulated by pIC in Atlantic cod macrophages at 24 and 48 HPS, compared with time-matched controls (Fig. 7I). The microarray results of *vimentin*, *grp94* and *catalase*, as candidate down-regulated transcripts at 24HPS, were not validated by qPCR assays. While the expression of *vimentin* was not changed by pIC, this transcript was significantly down-regulated in both groups at 48 HPS compared with 3, 6 and 12 HPS (Fig. 7F). While stable expression was recorded for *grp94* in the pIC group over time, this transcript was up-regulated in the control group at 12 h compared both to other sampling times in the control group and to the time-matched pIC group (Fig. 7G). This suggests that pIC inhibited the up-regulation of *grp94* in Atlantic cod macrophages at 12 HPS. The expression of *catalase* did not vary within the control group over time, but it increased within the pIC group at 48 HPS compared with 3, 6, and 24 HPS (Fig. 7H).

4. Discussion

The microarray results showed 446 differentially expressed probes in pIC-stimulated Atlantic cod macrophages in comparison with control macrophages at 24 HPS. After BLASTx aligning the contig sequences representing pIC-responsive probes against NCBI's nr database, 92 up-regulated and 99 down-regulated probes remained unannotated. The proportion of unannotated pIC-responsive transcripts in down-regulated probes (61.4%) was markedly higher than that of up-regulated probes (34.7%), similar to the results seen in a previously published study on the transcriptome profile of the antiviral response of Atlantic cod brain (Krasnov et al.,

2013). The pIC-responsive transcript list in our study was compared with the spleen transcriptome response of pIC-injected Atlantic cod [FO diet group; FDR < 0.01, fold change > 1.3; (Booman et al., 2014)], which was assessed using the same 20K microarray platform as that used in the current study; 160 microarray-identified probes in our macrophage study overlapped with pIC-responsive probes of the Atlantic cod spleen in the control diet group (see Table 2 and Supplemental Table S1). The hierarchical clustering analysis revealed that all pIC-stimulated macrophage samples clustered in a separate branch from all control macrophages (Fig 3); this was expected, since the transcripts included in this analysis were identified by SAM as pIC-responsive. The following discussion provides information on the genes and molecular pathways activated by pIC stimulation in Atlantic cod macrophages.

We identified transcripts encoding 5 receptors that play key roles in recognition of dsRNA in Atlantic cod macrophages (Fig. 4). The expression of macrophage *tlr3* increased in response to pIC at 24 and 48 HPS compared with time-matched controls, and this was similar to the response of Atlantic cod head kidney cells to pIC (Holen et al., 2012). An earlier induction by pIC (at 12 HPS) in cod macrophages was recorded for *tlr7*, and this transcript was also significantly up-regulated by pIC at 24 and 48 HPS (Fig. 4B). TLR3 and TLR7 are evolutionarily conserved PRRs that detect RNA molecules of pathogens in endosomes (Akira et al., 2006; Kumar et al., 2009). TLR3 is responsible for recognition of dsRNA and initiation of immune responses through the TRIF (TIR domain-containing adaptor protein inducing IFNB)-dependent pathway in mammals (Akira et al., 2006; Kumar et al., 2009; Yu and Levine, 2011) and fish (Palti, 2011; Workenhe et al., 2010). While TLR7 is known to recognize ssRNA and mediate the production of type I IFN through the MyD88 (myeloid-differentiation primary-response gene 88)-dependent pathway in mammals, its role is unknown in fish (Palti, 2011;

Zhang et al., 2014) (see Fig. 8 for pathways activated by dsRNA in Atlantic cod). In contrast to other *tlr*s, *tlr22g* was down-regulated by pIC in Atlantic cod macrophages at 24 HPS (Fig. 4C). TLR22 is known as a fish-specific PRR that can recognize dsRNA on cell surfaces (Palti, 2011; Zhang et al., 2014). Sundaram et al. (2012) showed that 12 different paralogues of Atlantic cod *tlr22* variably responded (i.e. time-, tissue- and paralogue-dependent responses) to a bath challenge of *V. anguillarum*. In previous studies involving the same 20K cod microarray platform as that used herein, *tlr22g* was not reported as differentially expressed in the spleen of pIC-injected cod (Booman et al., 2014; Hori et al., 2012); therefore, this transcript may have specific roles in pathogen recognition of Atlantic cod head kidney cells.

In agreement with a previous *in vitro* study on pIC stimulation of an Atlantic cod larvae cell line (ACL) (Jensen et al., 2013), *lgp2* and *mda5* were up-regulated at 24 and 48 HPS in the present study. The RLR family (consisting of RIG-I, LGP2 and MDA5) is involved in the detection of cytoplasmic RNA of viruses and the induction of transcription factors [e.g. IRF7/3 and NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1)] through interaction with MAVS/IPS-1 (mitochondrial antiviral-signalling protein) and the phosphorylation of TBK1 (TANK-binding kinase 1) and IKKs (NFKBIA kinase alias IKBK) (Akira et al., 2006; Takeuchi and Akira, 2008). The current study also revealed the over-representation of GO terms associated with the positive or negative regulation of the MDA5 signalling pathway among pIC-responsive transcripts. RIG-I and MDA5 are responsible for recognition of ssRNA and dsRNA, respectively, and initiation of signalling cascades; conversely, LGP2, lacking CARDs (caspase activation and recruitment domains), regulates (positively or negatively) or facilitates the activity of RIG-I and MDA5 (Kato et al., 2006; Takeuchi and Akira, 2008; Yu and Levine, 2011). Nonetheless, the function of LGP2 in fish remains controversial

(Zhang and Gui, 2012); for example, the over-expression of fish *lgp2* has been shown to promote the induction of IFN and ISGs in Japanese flounder, *Paralichthys olivaceus* (Ohtani et al., 2010) and rainbow trout, *Oncorhynchus mykiss* (Chang et al., 2011), but to negatively regulate pIC-induced activity in goldfish, *Carassius auratus* (Sun et al., 2011b). Previous studies from our lab have reported *lgp2*, but not *mda5*, as a responsive transcript in spleen of pIC-injected cod (Booman et al., 2014; Hori et al., 2012; Rise et al., 2008). The function of *mda5* in pIC recognition and signalling has been described in mammalian macrophages (Semple et al., 2015). Similarly, teleost MDA5 (i.e. Japanese flounder) was shown to play an important role in the pIC-dependant signalling pathway (Zhou et al., 2014). Our findings indicate the importance of *mda5* in antiviral immune responses of cod macrophages.

pIC-responsive transcripts involved in signal transduction and transcription (e.g. *stat1*, *nfkbia*, *pkr*, *irf1*, *irf7*, *irf10* and *znfx1*) were up-regulated in macrophages as in previously conducted studies on the spleen transcriptome response of Atlantic cod injected with either pIC or formalin-killed atypical *A. salmonicida* (ASAL) (Booman et al., 2014; Hori et al., 2012; Inkpen et al., 2015; Rise et al., 2008) or the brain transcriptome profiling of nodavirus carrier and/or pIC-stimulated Atlantic cod (Rise et al., 2010). As shown in Fig. 8, upon stimulation of TLRs and activation of several proteins (e.g. PKR), the IKK multiprotein complex phosphorylates NFKBIA, leading to the expression of inflammatory cytokines (reviewed by Kawai and Akira, 2007; Yu and Levine, 2011). The microarray analysis in this study showed a 2-fold increase in expression of *pkr* (dsRNA-dependent protein kinase) by pIC. In addition, we observed the over-representation of GO terms related to the positive regulation of NFKB1 transcription and import into the nucleus in the pIC-responsive transcript list, thus suggesting the possible role of this transcription factor in antiviral immune responses of Atlantic cod

macrophages. As proposed by Hori et al. (2012) with regard to the pIC response of cod spleen, the slight but significant up-regulation of *nfkbia* observed in the present study could have occurred as a result of degradation and re-synthesis of NFKBIA in pIC-stimulated Atlantic cod macrophages (see Fig. 8). The up-regulation of z*nfx1* by pIC as seen herein suggests the potential role of this transcript as a mediator of immune-related pathways (e.g. NFKB1) in cod macrophage immune responses. Although z*nfx1* has been reported as a pIC-responsive transcript in fish (Krasnov et al., 2011; Rise et al., 2010), its function in fish remains undetermined.

We identified *irf1, irf7* and *irf10* as pIC-responsive transcripts in Atlantic cod macrophages. IRFs (e.g. IRF3 and IRF7) bind to interferon-sensitive response elements (ISREs) and increase the production of IFNs and ISGs (Akira et al., 2006; Bonjardim et al., 2009). In previously published studies, the highest expression of *irf7* and *irf10* was found in spleen of pIC-injected cod at either 6 (Rise et al., 2008) or 24 (Hori et al., 2012) hours post-injection. Both *irf7* and *irf10* were significantly up-regulated by pIC at 12 HPS (7.1- and 4.2-fold, respectively), suggesting that these transcripts have important roles in early immune responses of Atlantic cod macrophages. The expression of *irf7* in cod macrophages peaked at 24 HPS (i.e. 10.6-fold up-regulation compared with time-matched control), but its expression lowered within the pIC group at 48 HPS. Goldfish *irf7* has been reported as a down-regulated gene in mature macrophages when compared with monocytes, indicating the important role of this transcription factor in early stages of macrophage development (Katzenback et al., 2013). However, a significant down-regulation of Atlantic cod *irf7* at the later time points (e.g. 48 h) was not seen within the control group of the current study. While *irf10* transcript expression response to pIC remained high at 48 HPS (comparable to that at 24 HPS), *irf7* transcript expression response to

pIC at 48 HPS dropped significantly (Fig. 5C,D). This suggests that *irf10* has a more important role than *irf7* in the later (i.e. 48 hours post-stimulation) response of cod macrophages to pIC.

In this investigation, *ifng* and *stat1* were up-regulated in the pIC group at 24 and 48 HPS, although the expression of *ifngr1* was not affected by pIC. The production of IFNs and ISGs elicit the antiviral immune response (Fig. 8). Following the binding of IFNG with IFNGR1/2, IFNG-activated factor (GAF, i.e. STAT1 homodimer) translocates into the nucleus and facilitates transcription initiation of IFNG-responsive genes by binding to the IFNG-activated sequence (GAS) (reviewed by Bonjardim et al., 2009; Sadler and Williams, 2008). The GO terms associated with the regulation of IFNG production and IFNG-dependent response were significantly over-represented in the pIC-responsive transcript list. A previously published study has shown that IFNG stimulates the nuclear localization of STAT1 in goldfish monocytes (Grayfer et al., 2010). Considering the inducibility of cod *stat1* by dsRNA observed in the present and previous studies (Booman et al., 2014; Hori et al., 2012), the IFN signalling in Atlantic cod appears to be mainly mediated via IFNG and STAT1.

The engagement of dsRNA with PRRs (e.g. TLRs and RLRs) activates the signalling pathways (e.g. TRIF-dependent pathway; see Fig. 8), and results in the up-regulation of ISRE-containing immune effectors (e.g. ISGs and IFNs) (Bonjardim et al., 2009; Langevin et al., 2013a). As in previously published studies of the spleen transcriptome response of pIC-injected cod (Booman et al., 2014; Hori et al., 2012; Rise et al., 2008), *isg15*s, *ccl13*, *mip2*, *viperin*, *sacsin*, *mig1*, *gig2*, *herc4*, *bty*, *iGTPase* (interferon-inducible GTPase), *ip44* (interferon-induced protein 44-like) and *mtap* (microtubule aggregate protein homolog isotype) were up-regulated in cod macrophages by pIC. Several well-known antiviral immune effectors conserved among vertebrates (e.g. *isg15*s, *viperin*, *ccl13*, and *mip2*) were identified in the current study by

microarray analysis, and subjected to time-dependent qPCR validation. All three paralogues of Atlantic cod *isg15* showed a strong up-regulation in macrophages after 24 h of pIC stimulation, peaking at 48 HPS (i.e. > 100-fold up-regulation compared with time-matched controls). Furnes et al. (2009) reported that the maximum response of all *isg15* paralogues in head kidney of Atlantic cod occurred 2 days after pIC injection. Mammalian ISG15, as a highly-conserved antiviral protein, hinders several viruses by attaching to the target protein in conjugation with other proteins [e.g. UBE1L (ubiquitin-activating enzyme E1-like) and HERC5], termed ISGylation (Zhang and Zhang, 2011). Additionally, mammalian ISG15 promotes the activation of some virus-responsive proteins such as PKR (Okumura et al., 2013). Likewise, zebrafish ISG15 has been demonstrated to inhibit RNA viruses, and promote the induction of *viperin* and *rig-I* (Langevin et al., 2013b). The present study shows the importance of *isg15* paralogues in antiviral immune responses of Atlantic cod macrophages.

The microarray-identified transcript list included 5 differentially expressed probes representing *viperin*. Consistent with the expression of *viperin* in ISAV-infected Atlantic salmon macrophage-like cells (Workenhe et al., 2009), the highest expression of *viperin* in pIC-induced Atlantic cod macrophages was recorded at 48 HPS (79-fold up-regulation). Similarly, the induction of *viperin* by pIC, chum salmon reovirus (CSV) and interferon-containing culture medium has been reported in monocyte/macrophage-like cells of rainbow trout at 24 HPS, suggesting that the viral induction of *viperin* in this species occurs via an IFN-dependent pathway (DeWitte-Orr et al., 2007). The virus-induced Viperin is chiefly activated by IRF1 or IRF3 in the RLR-associated pathway (Helbig and Beard, 2014). Similar to mammals, fish *viperin* can be triggered by dsRNA via a RLR-dependent pathway (Wang et al., 2014) and also by type I and II IFN (Aggad et al., 2009; Sun et al., 2011a). In addition to the inhibitory effects of Viperin

on virus replication, it facilitates the induction of IRF7 by interacting with signal mediators (e.g. recruiting IRAK1 and TRAF6 to lipid bodies), and enhances the TLR7- and TLR9-dependent pathogen recognition (Saitoh et al., 2011). The over-representation of GO terms associated with regulation of TLR7 and TLR9 signalling pathways in pIC-responsive transcripts of Atlantic cod macrophages can be explained by the possible role of Viperin in modulation of these PRRs.

In the current study, the GO term "positive regulation of chemokine production" was over-represented in pIC-responsive macrophage transcripts. Chemokines are a superfamily of small cytokines that act as chemoattractants, and regulate leukocyte migration and trafficking (reviewed by Mantovani et al., 2004). We observed a significant increase in *ccl13* expression by pIC at 24 and 48 HPS (26.6-fold at 48 HPS). In addition, *mip2*/*cxcl2* showed the strongest response to pIC in Atlantic cod macrophages (644.7-fold increase at 48 HPS). Mammalian MIP2, as a crucial chemokine for neutrophils, is secreted by different cells (e.g. macrophages, epithelial and intestinal cells), and is activated by viruses or LPS via IL-1R- and NFKB1-dependent pathways (De Plaen et al., 2006; Di Paolo et al., 2009). CCL13, classified as a MCP (monocyte chemoattractant protein) stimulates the expression of adhesion molecules and pro-inflammatory cytokines (Mendez-Enriquez and García-Zepeda, 2013). *ccl13* and *mip2* have been identified in some fish species such as zebrafish (Baoprasertkul et al., 2005; Peatman and Liu, 2006), but their functions in fish are yet to be determined.

Some pIC-responsive transcripts (e.g. *herc4*, *gig2*, *bty*, and *sacsin*) identified in Atlantic cod macrophages, may be categorized as fish-specific immune effectors, since they were previously shown to be virus- or pIC-responsive transcripts in Atlantic cod and/or other fish species (Booman et al., 2014; Wang et al., 2013; Workenhe et al., 2009), but they are not known as virus- or dsRNA-responsive transcripts in higher vertebrates. There was a strong up-

regulation of *herc4* in pIC-induced Atlantic cod macrophages (38-fold increase at 48 HPS), as previously seen in ISAV-infected Atlantic salmon macrophage-like cells (Workenhe et al., 2009). However, *herc4* was reported as a weakly down-regulated transcript in the brains of nodavirus carrier Atlantic cod (Rise et al., 2010). Furthermore, *herc6* was identified herein as a pIC-responsive transcript in Atlantic cod macrophages by microarray analysis. HERC4 and HERC6 are members of a family of proteins having HECT and RCC1 domains. Some HERC family members are known to be important proteins in antiviral responses. For example, human HERC5 is established as the most recently evolved HERC family member and an IFN-induced protein that mediates the process of ISGylation by conjugating to ISG15 as the main E3 ligase, and inhibits viral replication (Woods et al., 2014). HERC6 is an E3 ligase for ISG15 in mice, which lack a HERC5 ortholog in their genome (Zhang and Zhang, 2011). Further investigations are needed to determine HERC4 or HERC6 function as an E3 ligase for ISG15 in lower vertebrates such as fishes. The strong induction of Atlantic cod *herc4* by pIC as seen herein suggests that this transcript plays an important role in antiviral immune responses of Atlantic cod.

We observed the significant up-regulation of *mig1* by pIC 12 h after pIC stimulation, peaking at 48 HPS. The highest induction of *mig1* in response to megalocytivirus infection was observed in different tissues (i.e. kidney, spleen and liver) of tongue sole (*Cynoglossus semilaevis*) at 48 h post-injection, although the peak of up-regulation for this gene occurred in virus-treated head kidney lymphocytes of this species at 2 HPS (Wang et al., 2013). Tongue sole *mig1* shares moderate similarity with *gig1* of several teleost species and VHSV-induced protein of rainbow trout (Wang et al., 2013). Indeed, the pIC-responsive probes identified as *mig1* in our study share significant similarity (e.g. as the second best BLASTx hit) with teleost *gig1* (e.g.

goldfish) or VHSV-induced protein of rainbow trout; therefore, further studies are needed to completely characterise these pIC-responsive transcripts. Goldfish *gig1* has been recently identified as a novel antiviral fish ISG that is highly responsive to pIC, IFN and UV-inactivated grass carp reovirus (GCRV) through RLR-dependent signalling (i.e. *mda5*, *rig-i*, *mita*, *tbk1* and *irf3*) and *stat1* (Sun et al., 2013; Sun et al., 2014). Similar to our results of *mig1*, the highest fold-induction of *gig1* and *gig2* in pIC-stimulated blastulae embryonic cells of goldfish was recorded at 24 and 48 HPS (Jiang et al., 2009; Sun et al., 2014). Moreover, our microarray analysis showed 2.36-fold increase in expression of *gig2* in pIC-induced cod macrophages at 24 HPS. Jiang et al. (2009) reported that goldfish *gig2* is a IFN- and pIC-inducible gene (i.e. harbouring ISREs and GASs in its promoter), and is activated by IRF7. Gene expression results of the present study, along with the over-represented GO terms (e.g. MDA5 signalling) in pIC-induced cod macrophages, indicate the role of this protein family in antiviral responses of Atlantic cod macrophages.

Several pIC-responsive *bty* transcripts were identified by microarray analysis. Further analysis revealed that these pIC-responsive probes of Atlantic cod *bty*s share between 80-94% similarity at the nucleotide level with a fully characterised Atlantic cod *bty* (Genbank: HM140849). The qPCR assays confirmed the microarray results for *bty-1* (probe ID: 40261; see Supplemental Table S1); this transcript was significantly up-regulated in a time-dependent manner after 12 h of pIC stimulation. The *bty-2* (probe ID: 44336; see Supplemental Table S1) microarray results were not validated by qPCR. Our bioinformatics analysis of Atlantic cod *bty*s, using BLAST against the EST and nt databases of NCBI revealed that the Atlantic cod genome encodes a large number of *bty* paralogues (data not shown). qPCR analyses were not performed for other microarray-identified *bty*s (e.g. probe ID: 36195 and 43099) because the qPCR primers

failed quality testing. Atlantic cod *bty* has been characterised as a pIC-responsive gene that encodes a tripartite motif (TRIM)-containing protein, and TRIM proteins have crucial functions in numerous biological processes including immune responses (Furnes and Robertsen, 2010). TRIMs are established as IFN-inducible proteins that can act like a PRR, inhibit viruses, and positively or negatively regulate immune signalling through the RIG-I-associated pathway (e.g. binding to CARD domain of RIG-I) and transcription factors (e.g. NFKB1 and IRF3) (McNab et al., 2011). Many functions of TRIMs are mediated by the activity of E3 ligases. It has been recently demonstrated that different members of virus-induced TRIMs in rainbow trout possess E3 ubiquitin ligase activity and affect antiviral signalling by ubiquitination (van der Aa et al., 2012). The microarray results of the present study suggest that different *bty* paralogues play roles in the antiviral immune response of Atlantic cod macrophages.

Similar to previous investigations on pIC-injected cod (Hori et al., 2012; Rise et al., 2010) and ISAV-infected Atlantic salmon macrophage-like cells (Workenhe et al., 2009), the microarray analysis in this study showed a strong increase in expression of *sacsin* (6.8-fold) by pIC, implying the role of this transcript in antiviral innate immunity pathways of Atlantic cod. Sacsin is documented as a protein involved in the central nervous system of mammals, although Atlantic salmon *sacsin* has been previously described as a pIC-responsive transcript with expression that is highly correlated with type I IFN (Krasnov et al., 2011). The precise role of sacsin in fish antiviral responses is not currently known.

In addition to known fish immune-related transcripts (e.g. *mig1*, *gig2*, *bty* and *tlr22g*), the present investigation identified several pIC-responsive cod macrophage transcripts that have known functions in mammalian immune responses or macrophage activity but poorly understood functions in fish. This included *cd9*, *mapk14*, *klfr*, *tap1*, and *optineurin*. In mammals, *cd9* is

involved in the regulation of cell adhesion, migration and MAPK activation (Peddibhotla et al., 2013). MAPKs can regulate the induction of several transcripts and the inflammatory response in conjunction with nuclear factors (e.g. IRFs and NFKB1) (Arthur and Ley, 2013). *mapk14* was up-regulated by pIC in Atlantic cod macrophages (1.5-fold increase; Table 2), and it has been established as a suppressor of TNF in mammalian macrophages (Arthur and Ley, 2013). Further, *klf4* (*krueppel-like factor 4*), a microarray-identified pIC-responsive transcript in Atlantic cod macrophages (1.2-fold up-regulation; Table 2), is an IFNG-induced gene playing roles in regulation of signalling activation of mammalian macrophages (Feinberg et al., 2005). In the present study, an up-regulation was recorded for *tap1* (2-fold) and *optineurin* (1.6-fold) by pIC stimulation (Table 2). IFN-induced *tap1* is activated in mammalian macrophages via STAT1 and IRF1, and acts as a pivotal protein in antigen processing of MHC I (Brucet et al., 2004). *optineurin* has previously been described as an inhibitor of IFN production in dsRNA-induced mammalian cells (Mankouri et al., 2010).

As identified by microarray, Atlantic cod *vimentin* was down-regulated in pIC-exposed macrophages, although this result was not validated by qPCR. Since Vimentin is an intermediate filament protein that can act as a regulator of macrophage differentiation and adhesion (Beneš et al., 2006), the down-regulation of *vimentin* in Atlantic cod macrophages at 48 HPS within both groups could have occurred due in part to the reduced requirement for this protein in differentiated and adhered macrophages. The contradiction observed between the microarray and qPCR results (i.e. at 24 HPS) of *vimentin* or other transcripts (i.e. *traip, ccr7, bty-2* and *grp94*) in this study can be caused by several reasons as discussed in Booman et al. (2011). For example, it may be explained by misassembled contigs used for microarray design, or variations in the location of qPCR primers and microarray probes, although we generally attempted to place the

qPCR primers in the region of the transcript corresponding to informative microarray probes. Moreover, we used different fish for the microarray study and the time-dependent qPCR validation (see section 2.7. for details), and individual variation may have led to disagreement between qPCR and microarray results for some pIC-responsive transcripts.

Heat shock proteins (HSPs) can be involved in immune responses; for instance, they are involved in activation of macrophages and dendritic cells as well as the production of cytokines and chemokines (van Noort et al., 2012). In disagreement with the transcriptome response of ISAV-infected Atlantic salmon macrophage-like cells (Schiøtz et al., 2008), *hsp70* transcript was found to be up-regulated in pIC-induced Atlantic cod macrophages (Table 2). On the other hand, microarray analysis showed a significant down-regulation for *grp94* (alias *hsp90b1*) in cod macrophages at 24 HPS; this result was not validated by qPCR, where the down-regulation of *grp94* by pIC was only seen at 12 HPS compared with time-matched controls. GRP94 was reported to stimulate the NFKB1 pathway and nitric oxide generation in murine macrophages (Reed et al., 2003).

In this study, the GO terms associated with the oxidation-reduction process and oxidoreductase activity were under-represented in the pIC-responsive transcripts list. The oxidation-reduction (redox) molecules including nitric oxide (NO) and reactive oxygen species (ROS) are mainly produced in macrophages as a result of phagocytosis and cell stimulation, and they have important roles in pathogen (e.g. virus) eradication, apoptosis and the activation of signalling pathways (Wink et al., 2011). Reduced oxidative response by viral infection has been previously reported in human macrophages (Müller et al., 1990). In addition, a genomics survey of salmon macrophage-like cells response to ISAV has identified both up- and down-regulated transcripts involved in ROS metabolism (Schiøtz et al., 2008). Although they suggested an

inverse correlation between oxidative stress and viral replication, more studies are needed to determine the role of ROS in antiviral immune responses of fish macrophages.

As identified by microarray and validated by qPCR analysis, *ctsa* was down-regulated in Atlantic cod macrophages by pIC at 24 and 48 HPS. Cathepsins can be involved in cell death by degradation of anti-apoptotic agents (Repnik et al., 2012). However, the expression of *bcl-x1* was not influenced by pIC stimulation in our study. In contrast to this study, *bcl-x1*, an anti-apoptotic biomarker, was shown to have a tissue-specific response to pIC in Atlantic cod (Feng and Rise, 2010). These findings, along with the cell death results reported herein, suggest that pIC stimulation may not influence the apoptosis of Atlantic cod macrophages. A similar result was seen for *p67-phox* (i.e. a NADPH oxidase) in the current study. NADPH oxidases are JAK2/STAT-activated transcripts that play roles in phagocytosis of fish macrophages (Olavarría et al., 2013). Although we did not find any change in expression of Atlantic cod *p67-phox* over time, the time-dependent qPCR results of this investigation may be affected by cell differentiation and macrophage activation. For example, the expression of *li8* and *il1b* was up-regulated in both groups over time. These transcripts can influence macrophage response or activation (Ma et al., 2003).

5. Conclusion

The present study showed the induction of transcripts involved in MyD88- and TRIF-dependent pathways by pIC in Atlantic cod macrophages. Also, this study identified several potentially fish-specific pIC-responsive transcripts (e.g. *tlr22g*, *gig2*, *bty*) in Atlantic cod macrophages. Some pIC-responsive transcripts (e.g. *herc4*) identified in this study may have

fish-specific immune functions since their orthologues in higher vertebrates are not known as immune responsive genes. Moreover, the time-dependent qPCR assays indicated that the transcriptome response of Atlantic cod macrophages to pIC occurs 12 h after stimulation and peaks at 24 HPS or 48 HPS. Some transcripts (e.g. *il8*, *il1b* and *vimentin*) may play a role in macrophage maturation and activation, as their expression varied over time in the control group. We identified a large number of immune-relevant pIC-responsive genes that can be useful for improving our understanding of antiviral immune responses of Atlantic cod macrophages. However, we acknowledge that there may be differences between Atlantic cod macrophage gene expression responses to pIC versus their responses to pathogenic viruses. Further studies are required to structurally and functionally characterize the pIC-responsive transcripts of cod macrophages, to profile their transcriptome responses to pathogenic viruses, and to understand the correlation between their activation (e.g. cytokine expression) and antiviral immune responses.

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Figure legends

Fig. 1. GO term annotation of pIC-responsive transcripts. The charts illustrate the GO term distribution (Biological Process level 2) of up-regulated (A) and down-regulated (B) transcripts in Atlantic cod macrophages (SAM, FDR < 0.05) exposed to pIC at 24 HPS. The numbers between brackets indicate the percentage of probes with each GO annotation.

Fig. 2. The significantly enriched GO terms, reduced to the most specific terms. The bar chart depicts the percentage of pIC-responsive probes in Atlantic cod macrophages at 24 HPS (SAM, FDR < 0.05) that were annotated with each GO term (Biological Process and Molecular Function; FDR < 0.05).

Fig. 3. Hierarchical clustering analysis based on pIC-responsive transcripts. A: Clustering of the pIC and PBS (control) samples based on all pIC-responsive transcripts. **B:** Clustering of samples based on a subset of pIC-responsive transcripts involved in pathogen recognition (signaling pathway or regulation of RLR and TLR families); the transcript names are derived from the most significant BLASTx hit (E-value < 1.00E-05). Samples with the same coloured bar at the top of the figure belong to the same biological replicate.

Fig. 4. qPCR results for pIC-responsive transcripts involved in pathogen detection. Data are presented as mean ± SE. An asterisk indicates significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent the significant differences within a group over time. The fold-change (pIC/control) values for each time point are shown below the figures. Significant differences were observed for all of the represented transcripts [*tlr3, tlr7, lgp2* and *mda5* ($p < 0.0001$); *tlr22g* (time: $p < 0.0001$ and treatment: $p < 0.05$)].

Fig. 5. qPCR results for pIC-responsive transcripts involved in signal transduction/transcription control. Data are presented as mean ± SE. An asterisk indicates significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent the significant differences within a group over time. The fold-change (pIC/control) values for each time-point are shown below the figures. Significant differences were observed for all of the represented transcripts [*stat1*, *irf7*, *irf10* and *znfx1* ($p < 0.0001$); *nfkbia* ($p < 0.05$); *traip* (time: $p < 0.0001$); *ccr7* (time: $p < 0.05$); *ifngr1* ($p < 0.05$)] except for *bcl-x1* ($p = 0.37$).

Fig. 6. qPCR results for pIC-responsive transcripts encoding well-known immune effectors. Data are presented as mean ± SE. An asterisk indicates significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent the significant differences within a group over time. The fold-change (pIC/control) values for each time point are shown below the figures. Significant differences were observed for all of the represented transcripts [*isg15-1*, *isg15-2*, *viperin* and *ccl13* ($p < 0.0001$); *isg15-3* ($p < 0.05$); *ifng* ($p < 0.01$), *il8* ($p < 0.05$); *il1b* (time: $p < 0.0001$ and treatment: $p < 0.05$)] except for *p67-phox* ($p = 0.46$).

Fig. 7. qPCR results for pIC-responsive transcripts encoding putative immune effectors. Data are presented as mean ± SE. An asterisk indicates significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent the significant differences within a group over time. The fold-change (pIC/control) values for each time point are shown below the figures. Significant differences were observed for all of the represented transcripts [*mip2*, *mig1*, *bty-1* and *herc4* ($p < 0.0001$); *vimentin* (time: $p < 0.001$); *grp94* ($p < 0.05$); *catalase* (time: $p < 0.05$); *ctsa* (time: $p < 0.001$ and treatment: $p < 0.0001$)] except for *bty-2* ($p = 0.053$).

Fig. 8. The signalling pathways stimulated by pIC in Atlantic cod macrophages. This figure was adapted from known mammalian pathways (Akira et al., 2006; Bonjardimet al., 2009; Yu and Levine, 2011). A question mark beside the gene/protein name indicates that it was not found in the Atlantic cod genome using nominal search in the Ensembl annotation of the Atlantic cod genome (http://ensembl.org/Gadus_morhua). A question mark below or above the gene/protein name indicates unknown immune function and signalling activation in mammals. The pIC-responsive genes identified in the present study are shown in italics. MDA5 (melanoma differentiation-associated protein 5), RIG-I (retinoic acid-inducible gene), MAVS (mitochondrial antiviral-signaling protein), FADD (FAS-associated death domain), RIP1 (receptor-interacting protein1), IKK (NFKBIA kinase), NFKBIA (NF-kappa-B inhibitor alpha), NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), TNF (tumour necrosis factor), IL (interleukin), MIP2 (macrophage inflammatory protein 2), CCL13 (C-C motif chemokine 13), IFN (interferon), NEMO (NFKB1 essential modulator or IKKG), TLR (Toll-like receptor), TRIF (TIR domain-containing adaptor protein inducing IFNB), TRAF (TNF receptor-associated factor), TANK (TRAF family member-associated NFKB activator), TBK (tank-binding kinase), IRF (IFN regulatory factor), MAPK (mitogen-activated protein kinase), AP1 (transcription factor AP1), ISRE (IFN-sensitive response element), Peli1 (pellino E3 ubiquitin protein ligase 1), PKR (IFN-induced, double-stranded RNA-activated protein kinase), TAK1 [transforming growth factor beta (TGFB)-activated kinase 1], TAB (TAK1-binding protein), MyD88 (myeloid differentiation primary response gene 88), IRAK (IL-1 receptor-associated kinase), IFNGR (IFN-gamma receptor), JAK (Janus kinase), STAT1 (signal transducer and activator of transcription 1), GAF (IFNG-activated factor), GAS (IFNG-activated sequence), ISG (IFN-stimulated gene 15), LGP2 (RNA helicase LGP2), GIG2 (IFN-inducible protein gig2), MIG1

(megalocytivirus-induced protein 1), IP44 (IFN-induced protein 44-like protein), iGTPase (IFN-inducible GTPase), BTY (bloodthirsty), HERC4 (E3 ubiquitin-protein ligase herc4).

Table 1. qPCR primers

Transcript name		Primer sequence 5' to 3'	R^2	Amplification efficiency (%)	Amplicon size (bp)
Transcript of interest					
toll-like receptor 3 (*tlr3*)[c]	Forward	GCCTGGGTGGTGATTAAGAA	0.990	85.9	151
	Reverse	GTCCCCAGTGAAGAGCTGTC			
toll-like receptor 7 (*tlr7*)[c]	Forward	AACAAGTAGCGGATGGAAGC	0.997	99.2[e]	164
	Reverse	ATGTCCAGGGAGACGCTGTA			
toll-like receptor 22g (*tlr22g*)[c]	Forward	GCAGTTCTTCATCACGTAGCC	0.994	97.8	200
	Reverse	ACGGAACCAGAGATCACCTT			
RNA helicase lgp2 (*lgp2*)[a]	Forward	ACAGAAGCCATCGCAGAAAT	0.994	85.5	105
	Reverse	TTTTGCAGCACGAATCAAAC			
melanoma differentiation-associated protein 5 (*mda5*)[c]	Forward	GGGAACATGGATCAAGAGGA	0.996	91.7	129
	Reverse	GGACACCTCAGACGGACTGT			
signal transducer and activator of transcription 1 (*stat1*)[a]	Forward	GCCAATGCCATGTGTTTATG	0.998	90.2	100
	Reverse	ACCTGGAGCAGTTCGTCAGT			
NF-kappa-B inhibitor alpha (*nfkbia*)[a]	Forward	GCCAGCAACTGATAAAGCATC	0.994	84.9	132
	Reverse	GGTCACAGAGGGAGACAGAAAA			
interferon regulatory factor 7 (*irf7*)[b]	Forward	CATGTGCTTTGGGGAGAAGT	0.988	85.3	152
	Reverse	TCTGTAGGCTGACGTTGGTG			
interferon regulatory factor 10 (*irf10*)[b]	Forward	CCGAGAAGCCCAATAAACTG	0.997	94.9	143
	Reverse	ATACTCCTCGCCAAAGCAGA			
NFX1-type zinc finger-containing protein 1 (*znfx1*)[a]	Forward	ATGCCACTATCGGTGGACAGA	0.999	81.9[e]	108
	Reverse	TCAACAGATTATTGCCCTCGG			
TRAF-interacting protein (*traip*)[c]	Forward	AGCCTACTGCACCATTTGCT	0.990	92.2[e]	108
	Reverse	GCTTGGAACCACTGAAGGAG			
interferon gamma receptor 1 (*ifngr1*)[d]	Forward	CAGCGACCATGAAACTCTGA	0.998	95.5	137
	Reverse	TGTAGTCGTTGTCGGGACTG			
CC chemokine receptor 7 (*ccr7*)[c]	Forward	CAGCACCTGTAGCCCTTACC	0.989	90.9	120
	Reverse	GCGCTGTAGCAGAAGGTCAT			
bcl-x1[d,f]	Forward	AGGTGTTCAGGGACAGCATC	0.997	81.9[e]	157
	Reverse	CAGTGGTCAATGTGGTCGTC			
interferon stimulated gene 15-1 (*isg15-1*)[a]	Forward	AGGACCAACAAAGGCTGATG	0.998	91.4	110
	Reverse	CAGCCGTCCGTTAAGGTAGA			
interferon stimulated gene 15-2 (*isg15-2*)[c]	Forward	GAGCCCAACAACATCCAAGT	0.998	87.8	165
	Reverse	GTCCAACTGCTTGCCTTCAT			
interferon stimulated gene 15-3 (*isg15-3*)[c]	Forward	CAAGAGTCGCACCTACACCA	0.992	97.8	164
	Reverse	CCGCGCTCACATTATAGGAC			
interferon, gamma (ifng)[d]	Forward	TCGCTCTTCATGTTGGTCTG	0.993	89.9	121
	Reverse	GGCCTTTCTGTGGATGTTGT			
viperin[a]	Forward	TGTTTCCACACAGCGAAGAC	0.998	88.4	108
	Reverse	TCCGCCAGAGAAGTTGATCT			
C-C motif chemokine 13 (*ccl13*)[c]	Forward	TGTGCCTCATCCAACTCAGA	0.996	88.1	162
	Reverse	AAACCTCTGCATCGTCATCC			
interleukin 8 (*il8*)[d]	Forward	ATCGGCTCCCTACTGGTTCT	0.997	80.1	125
	Reverse	ATGTGACGACCAATCTGACG			
interleukin 1, beta (il1b)[d]	Forward	AACACGGACGACCTGAAAAG	0.999	83.4	126
	Reverse	GCTGATGTACCAACCGGAGT			
NADPH oxidase cytosolic protein p67phox (p67-phox)[d]	Forward	ATAGTAGGCCCCTCGTCCAC	0.996	83.5	168
	Reverse	AGCTGTGGTCCAATCCAAAG			
macrophage inflammatory protein 2 (*mip2*)[a]	Forward	GTGTTTCCAGCAGATCACTCG	0.998	90.9	118
	Reverse	TGTTCCACTTGGTGAGGAGTC			
megalocytivirus-induced protein 1 (*mig1*)[c]	Forward	CAGTCCTGGTTTGGTGGTCT	0.998	80.8[e]	132
	Reverse	GCTTCCTCTGAAACCCAACA			
bloodthirsty-1 (*bty-1*)[c]	Forward	GGAGACCAGACCTGCATCAT	0.994	93.5	153
	Reverse	ATCCCCTTGACCCCTAAGAA			
bloodthirsty-2 (*bty-2*)[c]	Forward	ATAGCAACCTGCTGGGATGT	0.990	82.6	171
	Reverse	GATCCGAAGGTCTGGTTCAA			
E3 ubiquitin-protein ligase herc4 (*herc4*)[c]	Forward	CGCTCTGTGGATTTGCTACA	0.989	87.8[e]	116
	Reverse	CCTTCTCTTGAGGGGAAACC			
vimentin[c]	Forward	AGAGCAGGATTTCCGTTCCT	0.995	83.5	165
	Reverse	CATGGTTCTGTGTGGACTCG			
heat shock protein 90, beta, member 1 (*grp94*)[c]	Forward	GCCTACCAGACAGGCAAAGA	0.997	86.4	115
	Reverse	CGCTCGCTACTCTCTTGAGC			
catalase[c]	Forward	GCGTGCTACCTGGTCAAGAT	0.997	83.3[e]	145
	Reverse	GACTACGGAAAGCAGGTCCA			
lysosomal protective protein (*ctsa*)[c]	Forward	AGGTCCTCCACAAACCACTG	0.996	85.6	125

	Reverse	ACCTACCCAACGATGAAGGA			
Normalizers					
eukaryotic translation initiation factor 3 subunit (*eif3*)[c]	Forward	AACTGTCCGTAGTCCGCAAG	0.995	91.1	125
	Reverse	CTGCTCAGCGAGAAACAGAA			
60S acidic ribosomal protein P1 (*rplp1*)[b]	Forward	TCTGAAGCTAAGGCCCTCAA	0.995	90.4	141
	Reverse	ATCGTCGTGGAGGATCAGAG			

[a] The primer sequences for these transcripts were previously published in Hori et al. (2012).
[b] The primer sequences for these transcripts were previously published in Inkpen et al. (2015).
[c] These primers were designed using ESTs or contigs representing the microarray probes.
[d] These transcripts were not present in the microarray significant gene list, and the primers were designed based on nucleotide sequence in NCBI.
[e] The amplification efficiencies of these primers were determined using4-point serial dilutions of cDNA.
[f] This primer pair was previously published in Feng and Rise (2010).

Table 2. Selected[1] immune-related probes differentially expressed between Atlantic cod macrophages in pIC or control groups at 24 HPS

Microarray probe ID	Name [species; GenBank accession number; E-value] [2]	Functional annotation [3]	Microarray fold-change [4]
38638	Macrophage inflammatory protein 2 precursor [*Esox lucius*; ACO13449.1; 5.25E-06]$_2$; (MIP2 alias CXCL2)	BP: chemotaxis, inflammatory response, immune response, cell chemotaxis; MF: chemokine activity	15.71
38607	Interferon stimulated gene 15-2 [*Gadus morhua*; ACZ02438.1; 4.05E-70]$_2$; (ISG15)	NA[5]	10.41
44624	Viperin [*Gadus morhua*; ADG85737.1; 3.33E-137]$_5$; (alias RSAD2)	BP: metabolic process; MF: catalytic activity, iron-sulfur cluster binding	9.85
43195	Megalocytivirus-induced protein 1 [*Cynoglossus semilaevis*; AFR33114.1; 9.94E-20]$_4$; (MIG1)	NA	8.53
38611	Interferon stimulated gene 15-3 [*Gadus morhua*; ACZ02439.1; 1.23E-72]; (ISG15)	NA	8.48
38359	Sacsin [*Chelonia mydas*; EMP28803.1; 3.98E-36]$_4$	MF: ATP binding	6.83
38617	Interferon-inducible GTPase_a [*Salmo salar*; ABW94983.1; 8.41E-69]; (iGTPase-a)	BP: metabolic process; MF: GTP binding, hydrolase activity, acting on acid anhydrides	6.19
44590	RIG-I C-terminal domain-containing protein 1 [*Gadus morhua*; ADG85724.1; 5.60E-101]$_{11}$; LGP2 (alias DHX58)	BP: metabolic process; MF: hydrolase activity, acting on acid anhydrides	5.72
44448	C-C motif chemokine 13-like precursor [*Takifugu rubripes*; NP_001266983.1; 6.15E-14]$_2$; (CCL13)	BP: immune response, cell chemotaxis; MF: chemokine activity	3.93
43797	Probable E3 ubiquitin-protein ligase HERC4-like [*Danio rerio*; NP_001139103.1; 7.06E-78]$_5$	BP: metabolic process, protein ubiquitination; MF: ubiquitin-protein transferase activity, ligase activity	3.43
38618	Interferon-inducible GTPase_b [*Salmo salar*; ABW94984.1; 1.30E-85]; (iGTPase-b)	BP: metabolic process; MF: GTP binding, hydrolase activity, acting on acid anhydrides	3.29
39321	NFX1-type zinc finger-containing protein 1 [*Dicentrarchus labrax*; CBN80799.1; 4.87E-22]$_8$; (ZNFX)	NA	3.27
38599	Interferon regulatory factor 10 [*Miichthys miiuy*; AHB59741.1; 5.19E-32]; (IRF10)	BP: regulation of transcription, DNA-templated; MF: regulatory region DNA binding, sequence-specific DNA binding transcription factor activity	3.11
42028	Secernin-3 [*Danio rerio*; NP_956032.1; 1.41E-95]	BP: proteolysis; MF: dipeptidase activity	2.90
38655	Interferon regulatory factor 7 [*Scophthalmus maximus*; ADQ52413.1; 6.69E-12]$_2$; (IRF7)	BP: regulation of transcription, DNA-templated, MF: regulatory region DNA binding, sequence-specific DNA binding transcription factor activit	2.79
43099	Bloodthirsty [*Gadus morhua*; ADM21462.1; 1.04E-42]$_{13}$; (BTY)	MF: zinc ion binding, metal ion binding	2.61
38565	Inducible heat shock protein 70 [*Miichthys miiuy*; CCF23009.1; 1.55E-173]; (HSP70)	BP: response to stress, MF: nucleotide binding, ATP binding	2.56
36483	CXC chemokine [*Scophthalmus maximus*; ACD62783.1; 1.20E-18]	BP: immune response, neutrophil chemotaxis, neutrophil degranulation; MF: chemokine activity	2.37
37853	Interferon-inducible protein Gig2 [*Carassius auratus*; AAP49829.1; 1.04E-36]	BP: metabolic process; MF: NAD+ ADP-ribosyltransferase activity	2.36
36407	CD9 antigen 2 [*Oplegnathus fasciatus*; BAM36395.1; 2.78E-34]	NA	2.32
39485	Helicase MOV-10 [*Salmo salar*; NP_001167174.1; 1.30E-45]$_4$	BP: metabolic process; MF: helicase activity	2.24
42596	Toll-like receptor 7 [*Larimichthys crocea*; AGO28200.1; 9.70E-26]; (TLR7)	BP: MyD88-dependent toll-like receptor signaling pathway, immune response, signal transduction, toll-like receptor 7 signaling pathway, defense response to virus; MF: single-stranded RNA binding, transmembrane signaling receptor activity	2.05
42754	TAP1 [*Oncorhynchus mykiss*; ABB52828.1; 2.77E-45]	BP: ATP catabolic process, transport, peptide transport, transmembrane transport; MF: nucleotide binding, ATP binding, peptide transporter activity, ATPase activity, ATPase activity, coupled to transmembrane movement of substances	2.03

40500	Polymeric immunoglobulin receptor [*Paralichthys olivaceus*; ADK91435.1; 2.94E-44]	NA	2.03
36933	Type 1 double stranded RNA activated protein kinase [*Gadus morhua*; ADG85735.1; 4.09E-69]; (PKR)	BP: protein phosphorylation, phosphorylation; MF: nucleotide binding, protein kinase activity, protein serine/threonine kinase activity, ATP binding, kinase activity	2.01
44545	Interferon regulatory factor 1 [*Gadus morhua*; ADG85733.1; 2.00E-120]$_4$; (IRF1)	MF: regulatory region DNA binding	1.95
44919	Immunoglobulin superfamily member 2, partial [*Chelonia mydas*; EMP37893.1; 3.60E-06]	NA	1.87
42622	TRAF-interacting protein [*Danio rerio*; NP_991170.1; 5.69E-44]; (TRAIP)	MF: zinc ion binding, metal ion binding	1.83
44541	Interferon induced with helicase C domain 1 [*Gadus morhua*; ADG85722.1; 1.65E-162]$_2$; alias Melanoma Differentiation-Associated Protein 5 (MDA5)	BP: metabolic process; MF: helicase activity, hydrolase activity, acting on acid anhydrides	1.83
46358	TNFAIP3-interacting protein 3 [*Myotis davidii*; ELK35575.1; 2.06E-08]	NA	1.79
44608	Toll-like receptor 3 [*Gadus morhua*; ADG85741.1; 2.81E-35]$_3$; (TLR3)	BP: signal transduction	1.78
45045	Ubiquitin carboxyl-terminal hydrolase CYLD, partial [*Ophiophagus hannah*; ETE61821.1; 2.06E-24]	BP: ubiquitin-dependent protein catabolic process; MF: hydrolase activity, ubiquitinyl hydrolase activity	1.68
44596	STAT1 [*Gadus morhua*; ADG85732.1; 8.24E-37]$_4$	BP: regulation of transcription, DNA-templated, signal transduction; MF: sequence-specific DNA binding transcription factor activity, signal transducer activity	1.64
48764	Interferon-induced protein 44-like protein, partial [*Epinephelus coioides*; AEA39725.1; 1.95E-16]$_2$; (IP44)	NA	1.64
38670	Optineurin [*Salmo salar* ; NP_001133761; 7.65E-30]	NA	1.63
39077	Microtubule aggregate protein homolog isotype-1 [*Paralichthys olivaceus*; BAK52806.1; 1.67E-67]; (MTAP)	NA	1.62
44530	NF-kappa-B inhibitor alpha [*Gadus morhua*; ADG85744.1; 5.08E-44]$_2$; (NFKBIA)	NA	1.58
38635	IL-4 receptor-2 precursor [*Oncorhynchus mykiss*; NP_001233265.1; 2.00E-14]	NA	1.55
36797	Cytotoxic and regulatory T cell protein precursor [*Oncorhynchus mykiss*; NP_001117972.1; 1.87E-52]	NA	1.53
38094	Probable E3 ubiquitin-protein ligase HERC6 [*Bos taurus*; NP_001179573.1; 3.47E-39]	NA	1.51
44572	Mitogen-activated protein kinase 14 [*Gadus morhua*; ADG85751.1; 0]; (MAPK14)	BP: MAPK cascade, protein phosphorylation, phosphorylation; MF: nucleotide binding, protein kinase activity, MAP kinase activity, ATP binding, kinase activity, transferase activity	1.51
36400	Tumor necrosis factor receptor superfamily member 5 precursor [*Salmo salar*; ACI69421.1; 2.66E-43]	NA	1.41
40882	Kinase C eta type [*Salmo salar*; ACN60220.1; 2.86E-136]	BP: phosphorylation, intracellular signal transduction; MF: nucleotide binding, protein kinase activity, protein serine/threonine kinase activity, ATP binding, transferase activity	1.41
43692	Tumor necrosis factor receptor superfamily member 14 precursor [*Osmerus mordax*; ACO09453.1; 1.57E-37]	NA	1.4
40316	Shugoshin-like 2 (S. pombe) [*Rattus norvegicus*; EDL99015.1; 8.44E-11]	NA	1.39
37678	Krueppel-like factor 4 [*Salmo salar*; ACI34008.1; 6.82E-22]; (KLF4)	MF: nucleic acid binding, metal ion binding	1.23
42605	Toll-like receptor 22g [*Gadus morhua*; AFK76491.1; 5.84E-63]; (TLR22g)	BP: signal transduction	0.78 (-1.27)
36119	BCL2/adenovirus E1B interacting protein 3-like a [*Danio rerio*; NP_001012242.1; 4.40E-73]	BP: positive regulation of apoptotic process, negative regulation of programmed cell death, defense	0.74 (-1.36)

		response to virus, mitochondrial outer membrane permeabilization	
37916	Immunoglobulin D heavy chain constant region variant b [*Gadus morhua*; AAF72569.1; 2.77E-135]	NA	0.73 (-1.37)
37913	Immunoglobulin light chain isotype 2, partial [*Trematomus bernacchii*; ABL60895.1; 5.07E-36]	NA	0.71 (-1.4)
40298	CC chemokine CK3 [*Sparus aurata*; ADE58986.1; 1.39E-12]	BP: immune response, cell chemotaxis; MF: chemokine activity	0.71 (-1.4)
36350	Catalase [*Oreochromis niloticus*; AEE40963.1; 4.76E-11]	BP: response to oxidative stress, oxidation-reduction process; MF: catalase activity, peroxidase activity, oxidoreductase activity, heme binding	0.69 (-1.43)
36377	CC chemokine receptor 7 [*Oncorhynchus mykiss*; NP_001268325.1; 3.78E-55]; (CCR7)	BP: Chemotaxis, inflammatory response, immune response, signal transduction, G-protein coupled receptor signaling pathway, chemokine-mediated signaling pathway; MF: signal transducer activity, G-protein coupled receptor activity, chemokine receptor activity, C-C chemokine receptor activity	0.68 (-1.47)
44976	Chymase precursor [*Esox lucius*; ACO13491.1; 2.71E-49]	BP: proteolysis , MF: catalytic activity , serine-type endopeptidase activity	0.66 (-1.5)
40296	Lysosomal protective protein [*Tupaia chinensis*; ELW66216.1; 2.08E-47] $_2$; (alias Cathepsin A; CTSA)	BP: Proteolysis; MF: carboxypeptidase activity, serine-type carboxypeptidase activity, peptidase activity, hydrolase activity	0.64 (-1.57)
37846	Vimentin [*Oncorhynchus mykiss*; NP_001118201; 9.20E-58]	MF: structural molecule activity	0.63 (-1.59)
37372	GRP94a protein [*Oncorhynchus mykiss*; CDG41617.1; 3.94E-61]; (alias HSP90b1)	BP: protein folding, response to stress; MF: ATP binding, unfolded protein binding	0.58 (-1.73)

[1] These differentially expressed probes were selected based on their function in immune response (i.e. involved in immune response or known to be an immune-responsive transcript in vertebrates). The probe identifiers (IDs) in bold indicate overlap between differentially expressed probes in the current study and previously published pIC-responsive transcripts in Atlantic cod spleen [(Booman et al. 2014); FO diet, FDR < 0.01, fold change >1.3]. See Supplemental Table S1 for the complete list of differentially expressed probes.

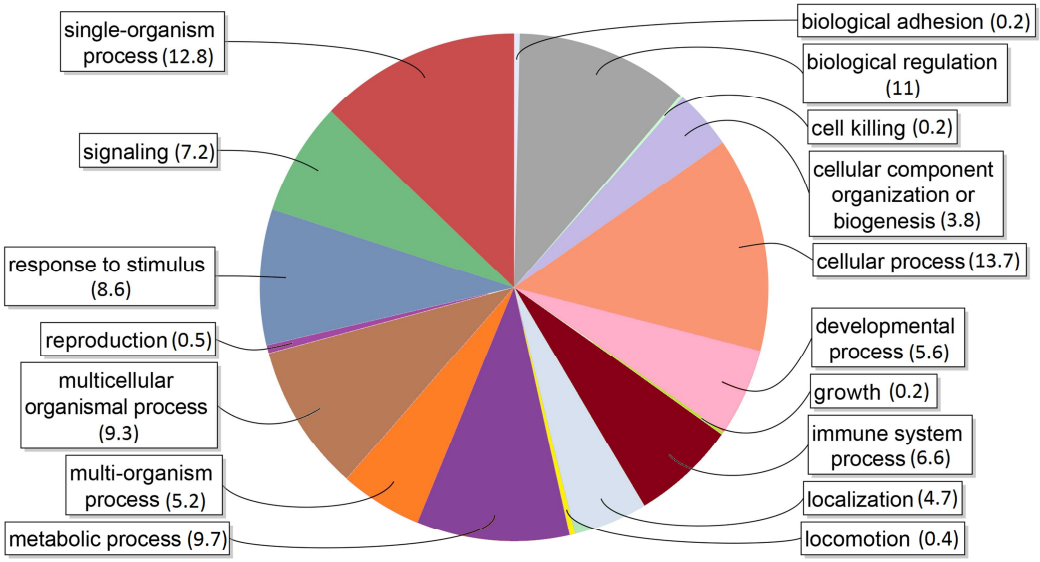
[2] Taken from the most significant (i.e. lowest E-value) BLASTx hit (not hypothetical, predicted and unnamed protein) in the Blast2GO annotation. The subscript after the BLASTx hit's name represents the number of differentially expressed probes sharing the same annotation (see Supplemental Table S1 for the complete list of differentially expressed probes).

[3] Functional annotation represents the GO (Gene Ontology) terms, including Biological Process (BP) and Molecular Function (MF), for the best BLASTx hit.

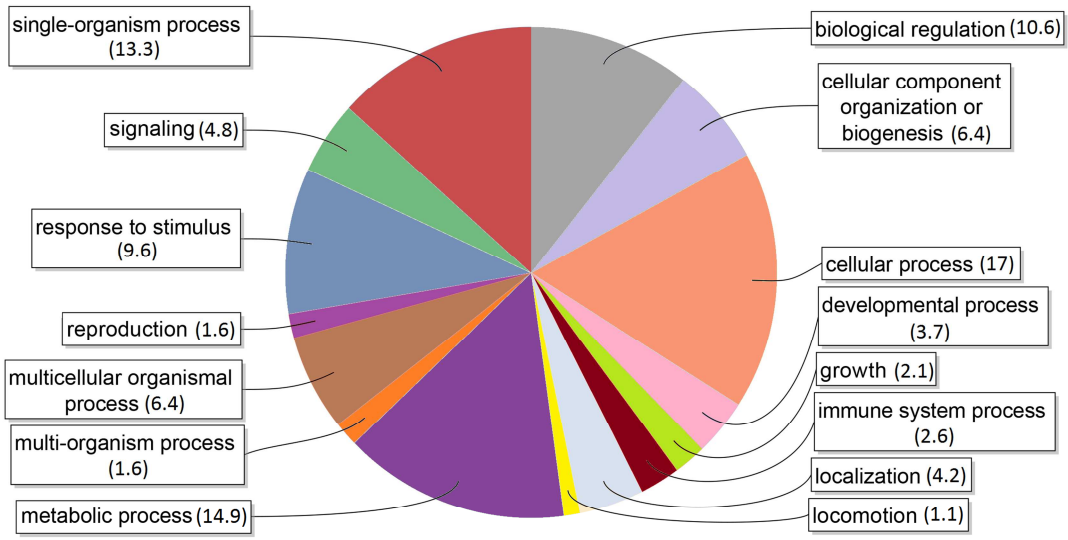
[4] Fold-change (pIC/control) for the differentially expressed probes (FDR ≤ 0.05) as outputted by SAM analysis. The numbers in parenthesis represent fold down-regulation calculated as the inverse of fold-change (i.e. 1/fold-change) for the values less than one.

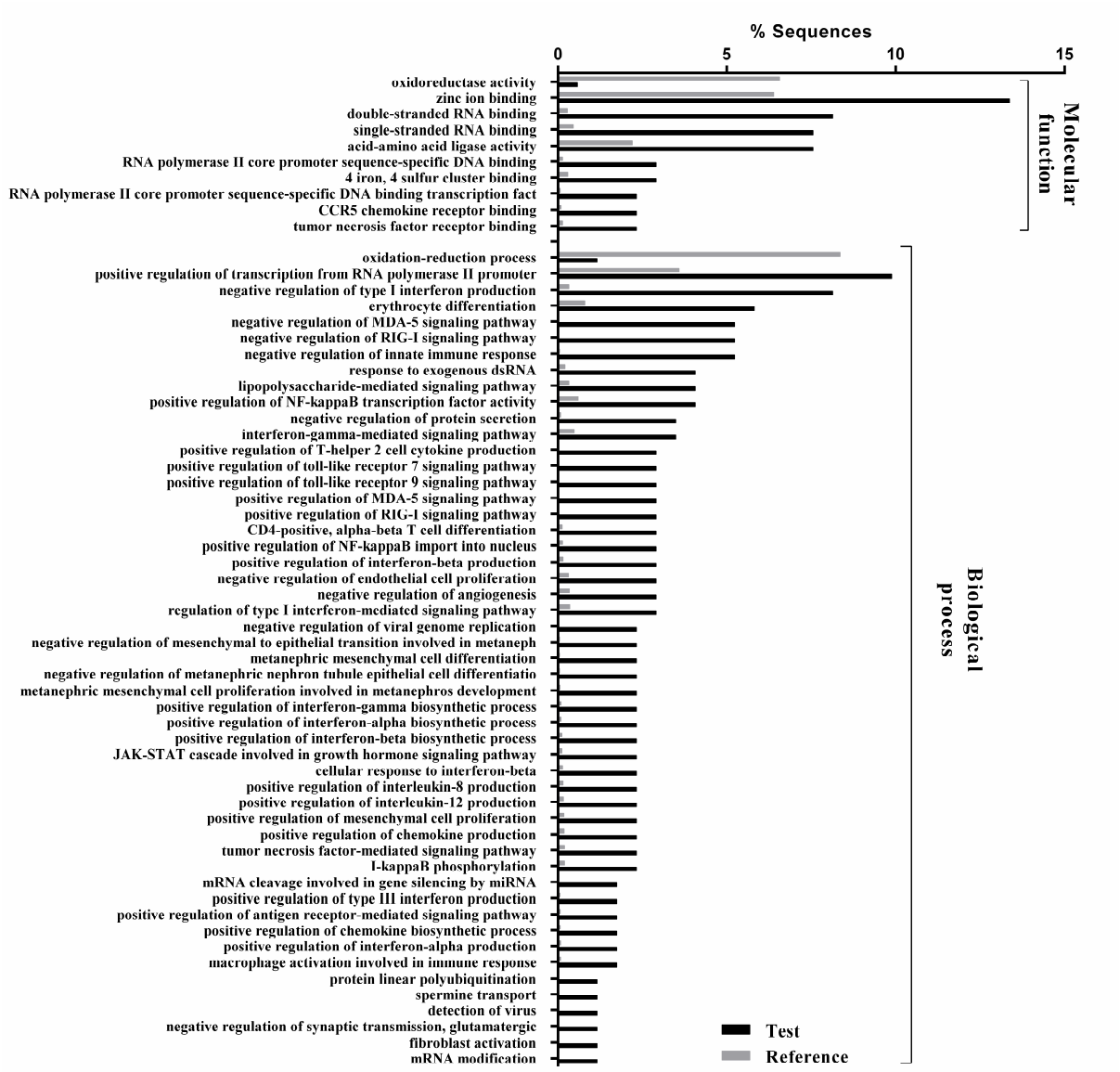
[5] Not available

A. Up-regulated genes

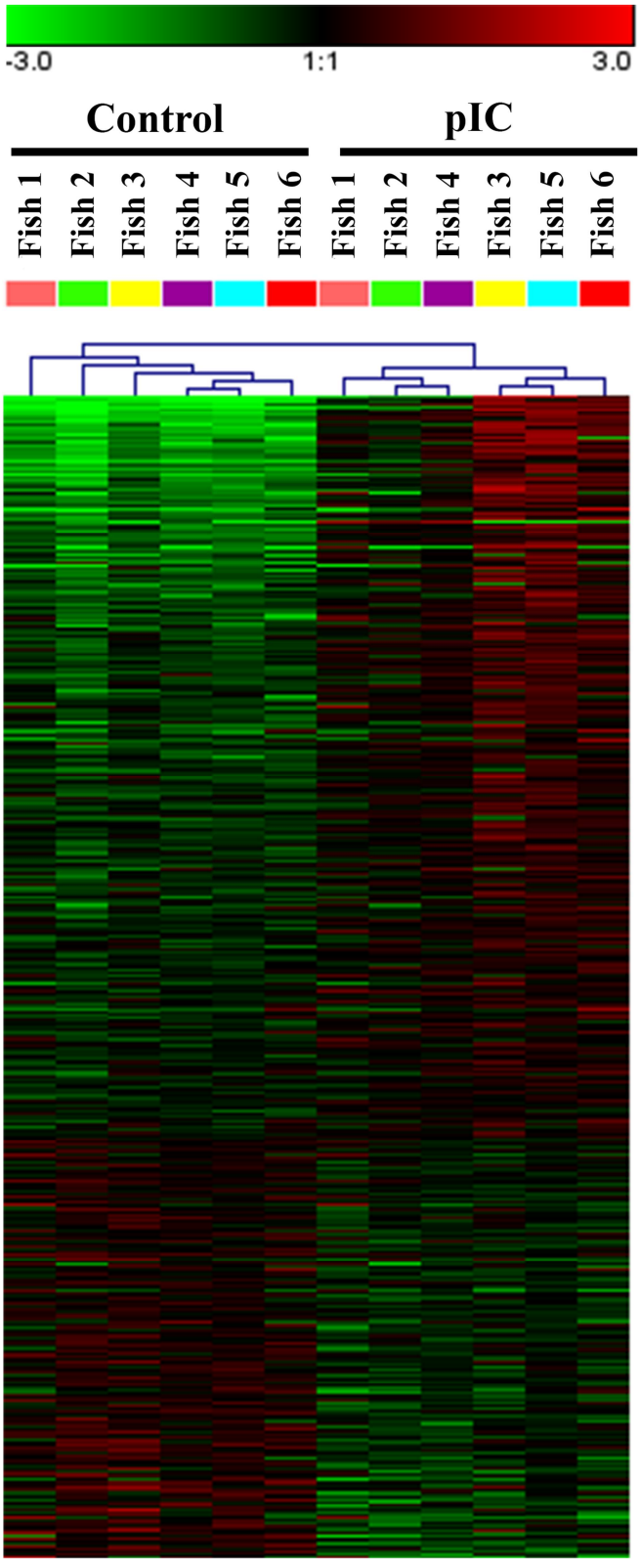


B. Down-regulated genes





A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Fish 6
Fish 1
Fish 2
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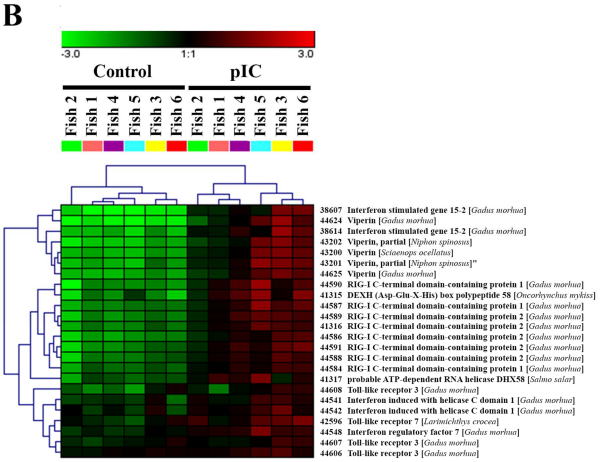
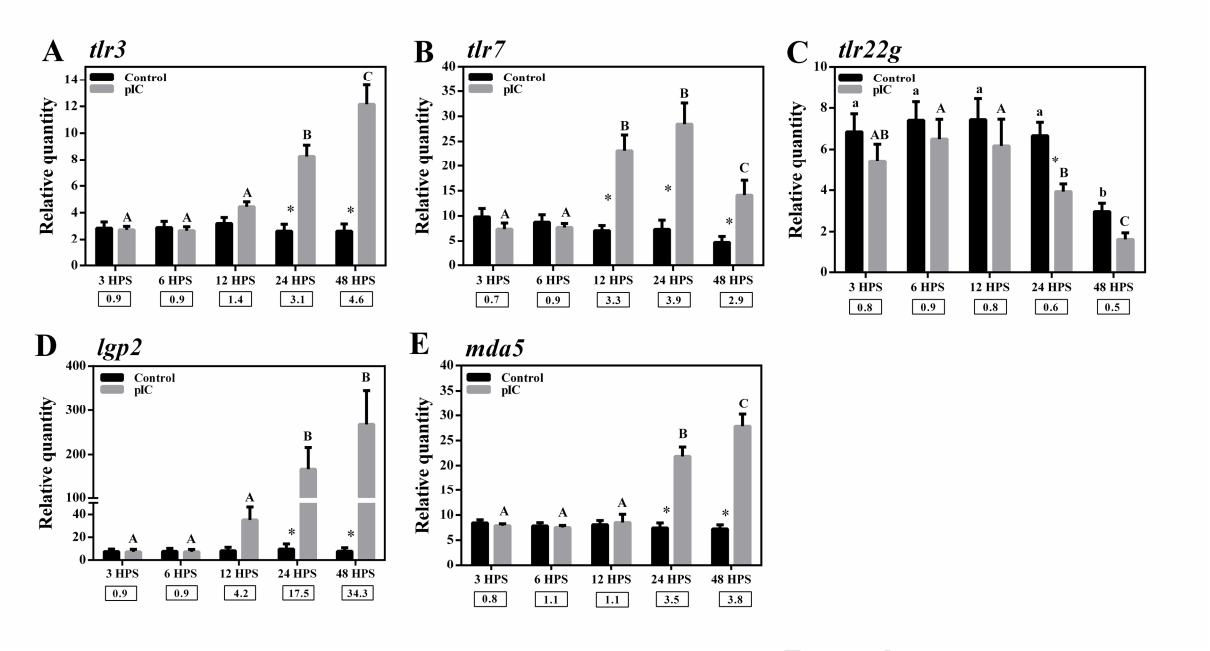
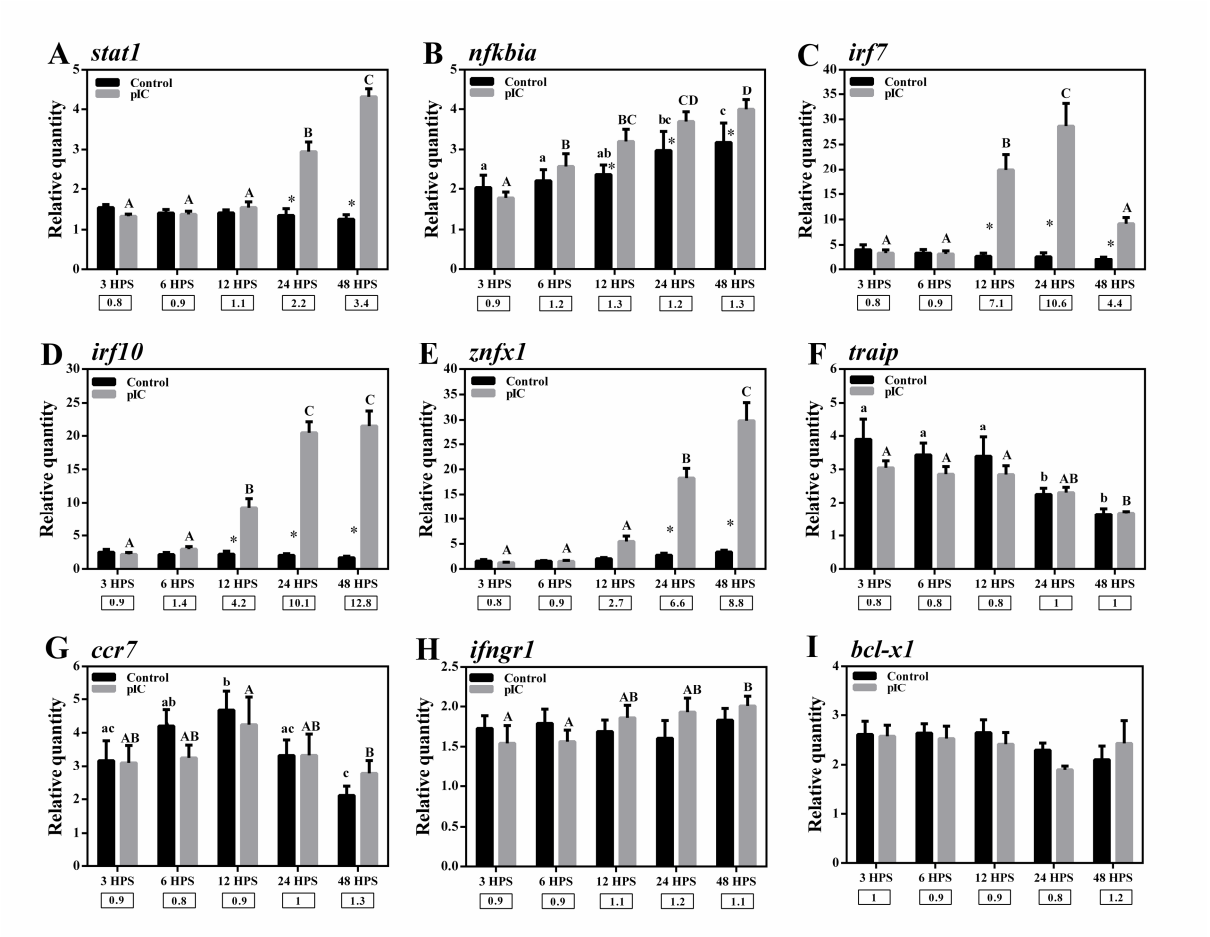
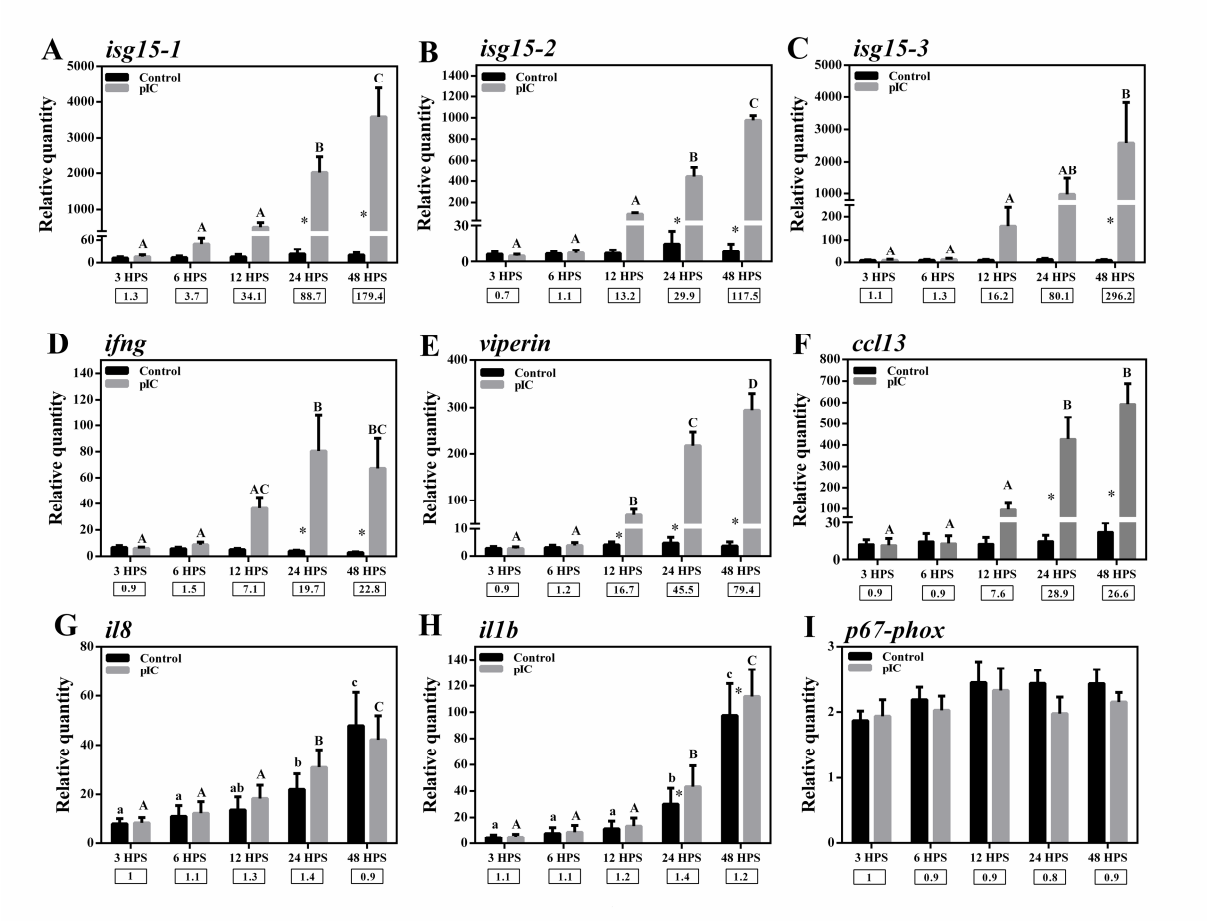
Fish 5
Fish 6

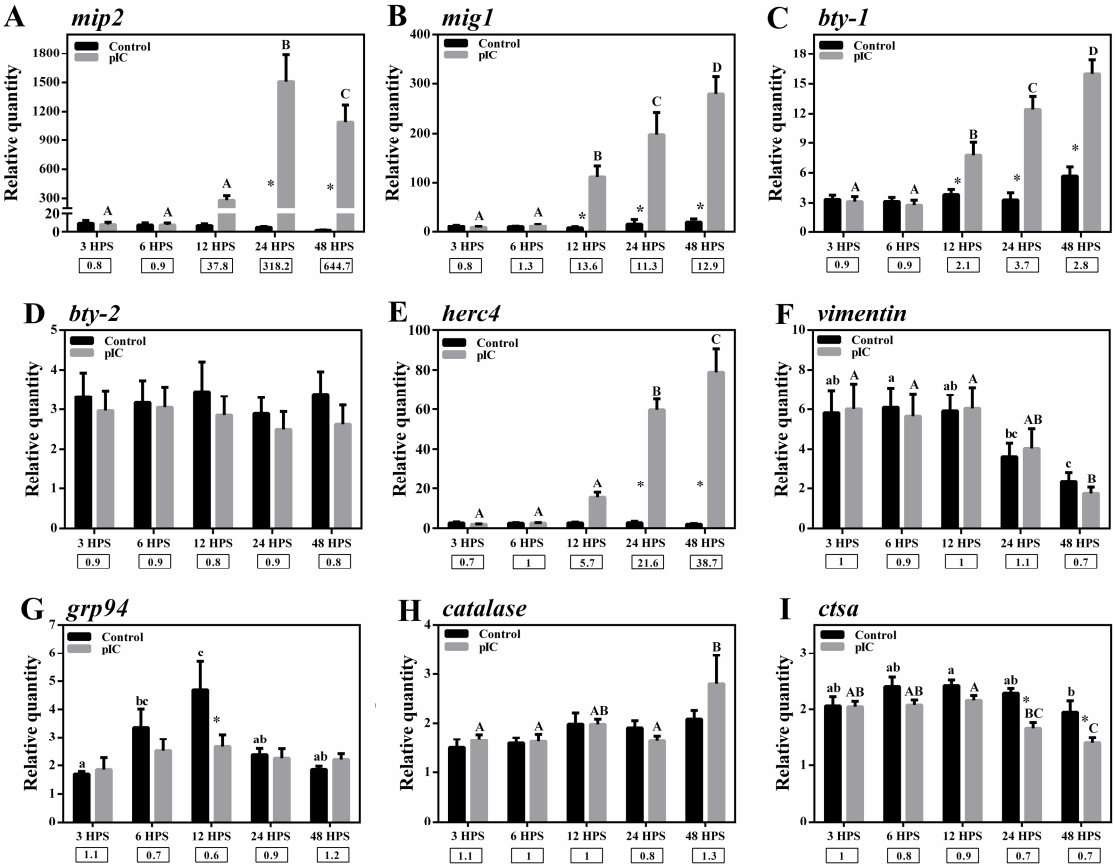
B

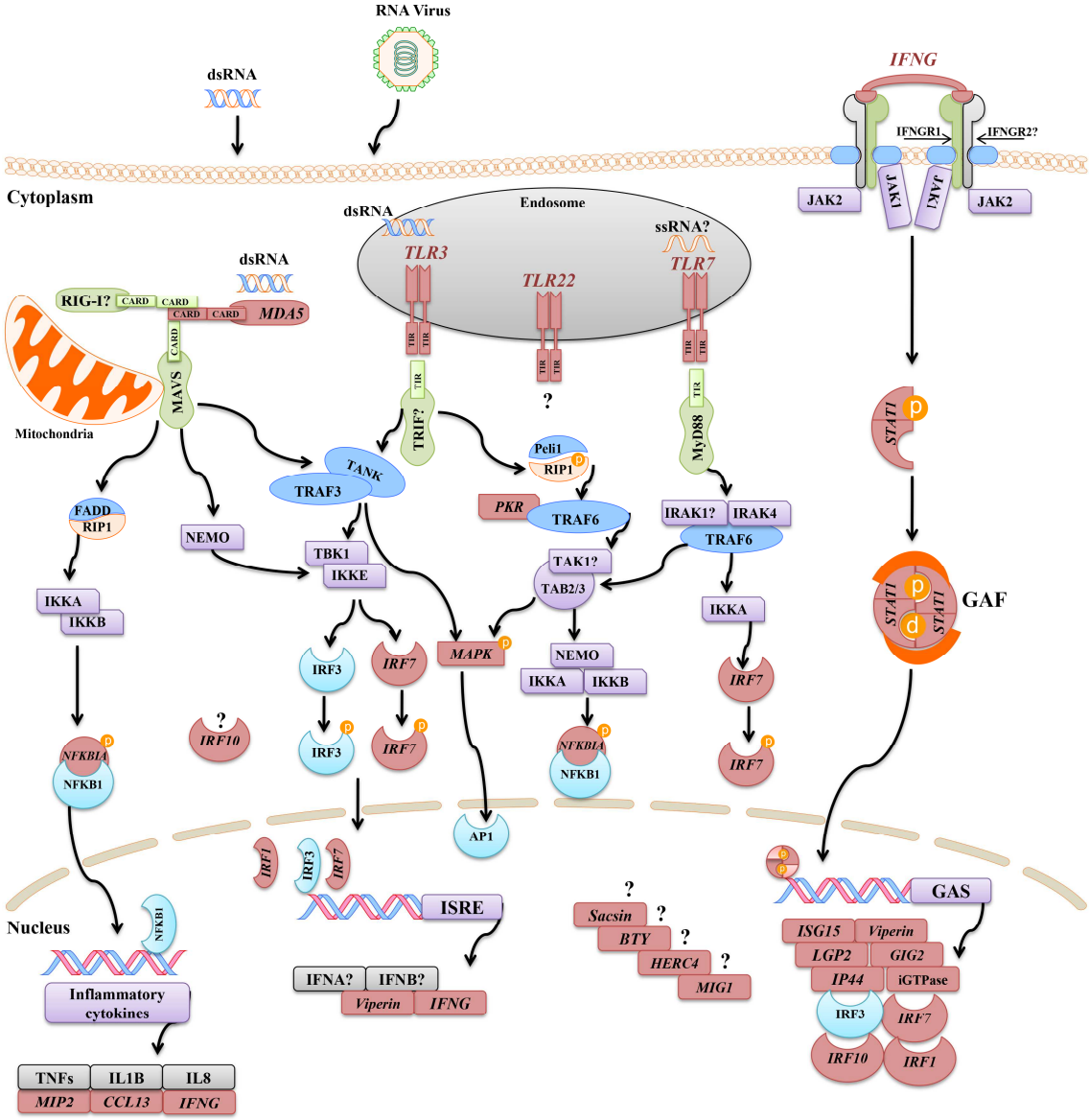











RNA Virus
dsRNA
IFNG
IFNGR1
IFNGR2?
JAK2
JAK1
TYK2
JAK2
Cytoplasm
dsRNA
Endosome
ssRNA?
TLR3
TLR22
TLR7
dsRNA
RIG-I?
CARD
CARD
CARD
CARD
MDA5
MAVS
Mitochondria
TRIF?
?
MyD88
STAT1
TANK
TRAF3
Peli1
RIP1
PKR
TRAF6
IRAK1?
IRAK4
TRAF6
FADD
RIP1
NEMO
TBK1
IKKE
TAK1?
TAB2/3
IKKA
IKKB
IKKA
IKKB
IRF3
IRF7
MAPK
NEMO
IKKA
IKKB
IRF7
?
IRF10
IRF3
IRF7
NFKBIA
NFKB1
NFKBIA
NFKB1
IRF7
STAT1
STAT1
GAF
AP1
IRF1
IRF3
IRF7
ISRE
Nucleus
NFKB1
Inflammatory cytokines
IFNA?
IFNB?
Viperin
IFNG
?
Sacsin
?
BTY
?
HERC4
?
MIG1
GAS
ISG15
Viperin
LGP2
GIG2
IP44
iGTPase
IRF3
IRF7
IRF10
IRF1
TNFs
IL1B
IL8
MIP2
CCL13
IFNG

Highlights

Cod macrophage transcripts that respond to viral mimic (pIC) identified by microarray.

Expression of 26 microarray-identified transcripts in pIC treated macrophages studied by qPCR.

pIC strongly induced expression of immune effectors (e.g. *isg15*, *viperin*, *herc4*, *mip2*).

pIC stimulation of cod macrophages occurs in a time-dependent manner, peaking at 24-48 h.

pIC (dsRNA) potentially activates MyD88- and TRIF-dependent signalling pathways in cod macrophages.