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Cloning and expression analysis of two ROR-γ homologues (ROR-γa1 and ROR-γa2) in rainbow trout *Oncorhynchus mykiss*

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Abstract

This paper describes the cloning and characterisation of two retinoid-related orphan receptor (ROR)-γ homologues (ROR-γa1 and -γa2) in rainbow trout (*Oncorhynchus mykiss*). The coding region predicted for both homologues consists of 1,410 base pairs (bp), which translate into two 469 amino acid (aa) proteins. The trout ROR-γs revealed a high conservation of both DNA- and ligand-binding domains (functional regions of the nuclear receptor family), and shared a high homology to mammalian ROR-γt. A phylogenetic tree containing ROR family members confirmed that both trout homologues clustered within the ROR-γ group. Both results suggested that these molecules are likely to be ROR-γ homologues, more similar to the mammalian splice variant ROR-γt than the full length ROR-γ. Expression analysis of tissues obtained from healthy fish revealed highest constitutive expression of trout ROR-γ in muscle, followed by the brain, heart and skin. This suggests that these genes may play an important role in such tissues. *In vitro* studies, using trout cell lines, demonstrated that ROR-γ is induced significantly by LPS and down-regulated by the presence of PolyI:C and recombinant interferon (IFN)-γ. Moreover, analysis of this gene in head kidney macrophages and mixed primary leucocyte cultures indicated that differences were apparent between the different cell types/sources used, indicating that its expression may be cell-type dependent. Additional studies to investigate the regulation of this gene *in vivo* demonstrated that its expression was significantly higher in vaccinated *vs* unvaccinated fish following bacterial (*Yersinia ruckeri*) challenge but it was down-regulated after a viral (VHSV) infection. This suggests a potential role of trout ROR-γ, a putative T_H17 transcription factor, in protection against extracellular bacteria.

Keywords: *Oncorhynchus mykiss*, ROR-γ, bacterial infection, vaccination, viral infection.

1. Introduction

Nuclear receptors (NRs) constitute one of the largest superfamilies of eukaryotic transcription factors, with over 60 family members known that share structural similarities [1,2]. They regulate the expression of genes involved in key cellular processes, such as cell growth, differentiation and apoptosis. These receptors function by promoting a link between signalling molecules and the transcriptional response upon binding of a variety of extracellular ligands [1-3]. The lipophilic ligands to which these receptors bind include a range of known hormones, from steroids (e.g. estrogens and progesterone) to thyroid hormones [3,4]. Since the activity of these receptors can be controlled by the direct action of natural and synthetic compounds, with their dysfunction resulting in disease, NRs are considered to be good targets for drug research [2,3]. Orphan nuclear receptors include the retinoid-related orphan receptors (RORs), whose ligands were unknown at the time they were identified and hence the reason they were named as "orphan" [2,3,5]. The ROR subfamily can be further divided into α, β and γ isotopes, also referred to as NR1F1-3 (Nuclear Receptor Nomenclature Committee), or RORA-C (Human Gene Nomenclature Committee) [6,7]. Members of this subfamily have been identified in several mammalian species, as well as in early vertebrates, such as bony fish [7,8]. In terms of structural organisation, ROR molecules share a similar structure with other members of the NR family, and contain the following functional regions: an N-terminal domain (A/B), a highly conserved DNA-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD) [7-9].

The highly variable N-terminal domain is present in all NRs. It contains a transcriptional activation function region, known as AF-1, which is recognized by coactivators or other transcription factors, acting in a ligand-independent manner [1, 10]. The DNA-binding domain, on the other hand, is highly conserved among the family members, and contains two C_4 zinc-finger motifs involved in the recognition of ROREs in the promoter region of the targeted genes [2,9]. ROR-γ receptors are known to bind preferably as monomers to these specific response elements

that consist of an AGGTCA motif preceded by an A/T rich-region [2,11]. This binding allows the receptors to perform their various roles through the communication between the receptor and its intracellular environment, regulating transcription [4,8]. DNA- and ligand-binding domains are connected together by a hinge region which is highly variable and flexible [2,9]. The ligand-binding domain is moderately conserved between members of the NR family and contains a second activation function region, described as AF-2. This region contains a characteristic motif, ΦΦXE/DΦΦ, where Φ corresponds to a hydrophobic residue and X can be any amino acid (aa), and acts as a ligand-dependent transcription factor [2,9].

In mammals, two isoforms of ROR-γ have been identified, and named ROR-γ1 and -γ2. The latter, commonly referred to as ROR-γt, lacks the A/B domain and thus consists of a truncated form of ROR-γ1, the result of alternative RNA splicing of a common transcript [9, 11-13]. They display different tissue distribution patterns and therefore regulate distinct physiological processes. A higher constitutive expression of ROR-γ1 was found in liver, muscle, thymus and kidney tissues [11,12,14]. In contrast, ROR-γt expression is restricted to a limited number of cell populations, such as developing thymocytes and T cells in secondary lymphoid tissues [7,13,15]. More recently this isoform was identified as the master T helper (T_H) 17 transcription factor, crucial for the differentiation of naïve $CD4^+$ T cells into activated T_H17 cells [16,17]. Most recently studies have revealed that ROR-γt is also expressed in a novel mucosal lymphocyte population in the intestinal lamina, which secretes IL-22 and co-expresses natural killer (NK) cell markers [8,19]. In mice and humans, ROR-γ molecules encode proteins of 516 and 518 aa, respectively, and share 88% aa homology [11,12,14]. In contrast, ROR-γt encodes an open reading frame of 495 aa in mice and 497 in humans [12,13]. In fish, ROR-γ homologues have been identified in zebrafish [8], with the characterisation of two isotypes of ROR-γ (ROR-γa and -γb). In the present study we report on the identification and characterisation of two ROR-γ homologues (ROR-γa1 and -γa2) in rainbow

trout, in order to gain a better insight into these molecules and their role in the immune system of early vertebrate species.

2. Material and Methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*), weighing approximately 100 g, were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, UK) and maintained in 1-m-diameter aerated fibreglass tanks supplied with a continuous flow of recirculating freshwater at 15 ± 1 ºC. Fish were fed twice daily on standard commercial pellets (EWOS), and were given a 2-week acclimatisation period prior to treatment.

2.2. Cloning and sequencing of ROR-γ

For the sequencing of trout ROR-γ homologues, a TBLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [20] was performed, identifying a rainbow trout expressed sequence tag (EST, GenBank accession number: **CX006390**), which shared 83% identity with the N-terminus of zebrafish (*Danio rerio*) ROR-γ [8]. The full-length cDNA sequence was obtained by RACE using SMART cDNA as described previously [21]. A single band was amplified by 5'-RACE PCR, using primers ROR-γR1 and -γR2, and cDNA samples obtained from spleen and head kidney. Sequence analysis revealed that the PCR products obtained from both tissues were identical, with sizes of approximately 664 bp, being 21 bp larger than the EST at the 5'-end. Amplification of spleen cDNA by 3'-RACE PCR, using ROR-γF1 and -γF2 primers, detected the presence of two bands. Homology analysis revealed that both products shared around 98% identity in the coding region, but only 78% in the 3'-untranslated region (UTR). This suggests that they may have arisen from two different paralogues, which are more closely related to the zebrafish ROR-γa, and were therefore designated as ROR-γa1 and ROR-γa2. Their nucleotide sequences

were submitted to the EMBL/DDBJ/GenBank nucleotide sequence database under the accession numbers: **FM883712** and **FM883713**, respectively. The sequences of all primers used are given in **Table 1**.

2.3. Bioinformatics

The generated sequences were analysed for similarity with other known molecules, and also used to identify homologous sequences in GenBank using the BLAST search [20]. Comparisons between more than two sequences were performed using the CLUSTAL W multiple sequence alignment package (http://align.genome.jp) [22] and conserved residues were shaded using the BOXSHADE server (version 3.21) (http://www.ch.embnet.org/software/BOX_form.html). Amino acid homology comparisons were performed using the MatGAT package (version 2.02) [23]. Phylogenetic trees were constructed from CLUSTAL W generated alignments using the Neighbour–Joining (N–J) method within the MEGA (version 4.1) package [24]. In addition, the domain structure was predicted using the SMART 6 program (http://smart.embl-heidelberg.de) [25].

2.4. Expression analysis

2.4.1. Tissue distribution

Six rainbow trout were killed and 14 tissues (liver, caudal kidney, spleen, heart, head kidney, skin, thymus, scales, brain, muscle, gonad, gills, tail fins and intestine) collected for RNA extraction using TRI Reagent® (Applied Biosciences) following the manufacturer's instructions. The first strand cDNA was synthesised using BioScript™ (Bioline, UK), diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20 °C. Real-time PCR was performed using IMMOLASE (Bioline) and SYBR Green fluorescent tag (Invitrogen) in a LightCycler® 480 System (Roche Applied Science, UK). The two trout ROR-γ molecules share high sequence

identity in the coding region that prevents the design of gene-specific primers for real-time PCR analysis. Thus a single pair of primers was designed in the coding region in order to detect the expression of both genes. To investigate if the transcript detected by those primers is the major product of trout ROR-γ, primers F3 and R3 were designed in the 5'-end in order to amplify any possible splice variants of the ROR-γ transcript. Real-time PCR analysis was performed in selected tissues (thymus, muscle, heart and brain). The relative expression of ROR-γ was calculated as arbitrary units and normalised against the expression level of rainbow trout elongation factor (EF)-1α, a house keeping gene. Primer sequences are listed in **Table 1**.

2.4.2. Maintenance of cell lines

Four rainbow trout cell lines derived from different tissues were used for this work: monocyte/macrophage-like RTS-11 cells from spleen [26], fibroblast-like RTG-2 cells from gonad [27], RTL from liver [28] and RTGILL from gill [29]. The latter was kindly supplied by the Virology group at Marine Scotland Science (Aberdeen). The cells were grown and maintained at 20 °C in Leibovitz L-15 medium (Gibco) supplemented with penicillin/streptomycin (P/S; 100 units/mL and 100 μg/mL, respectively; Gibco) and 30% foetal bovine serum (FBS; Biosera) for RTS-11, or 10% for all the other cell lines. Cells were counted using trypan blue (Sigma-Aldrich) and seeded at a concentration of 5-10 x 10^5 cells/mL into 25-cm^2 flasks (Sarstedt).

2.4.3. Preparation of primary leucocyte cultures

Head kidneys were collected aseptically from four fish, and cells were pushed through a 100-μm nylon mesh (John Stanier) with incomplete L-15 medium, i.e. L-15 supplemented with P/S, 0.5% FBS and 10 U/mL heparin (Sigma-Aldrich). The suspensions were then centrifuged at 200 g for 5 min, and washed once with complete medium (same constituents as incomplete medium but with 10% FBS). Cells were counted and 5 mL of 1-1.5x10^6 cells/ml were seeded into 25-cm^2 flasks ready for treatment.

2.4.4. Preparation of macrophage primary cultures

To obtain trout primary macrophage cultures, head kidney leucocyte suspensions, isolated as described previously, were resuspended in L-15 medium with 0.1% FBS and P/S. Five millilitres of cells were added into 25-cm^2 flasks, at a final concentration of 2 x 10^6 cells/mL. In order to allow the adherence of macrophages, the cells were left in an incubator at 20°C for 2 h. After incubation, the non adherent cells were removed by washing twice with L-15 medium in the same conditions. After the washing steps, the primary cultures were then incubated with complete medium (L-15 medium supplemented with 10% FBS and P/S) at the same temperature. After 3 days any non adherent cells were again removed before use.

2.5. Induced expression in vitro

2.5.1. Primary cultures

Head kidney primary cultures, prepared as described above, were incubated with known stimulatory concentrations [30] of *E. coli* lipopolysaccharide (LPS; 25 µg/mL), polyinosinic:polycytidylic acid (PolyI:C; 50 µg/mL), phorbol 12-myristate 13-acetate (PMA; 100 ng/mL), calcium ionophore (CI; 0.5 µg/mL), phytohaemagglutinin (PHA; 10 µg/mL), PMA (100 ng/mL) plus CI (0.5 µg/mL), dexamethasone (DM; 0.5 µg/mL), or left untreated (control), for 4, 8 and 24 h. All chemicals were purchased from Sigma-Aldrich. After the incubation periods, cells attached to the flasks and in suspension, were dissolved in TRI Reagent®. The RNA extraction and cDNA synthesis was performed as described above. Data were normalised to EF-1α gene expression and analysed against controls using the Pfaffl method [31].

2.5.2. Macrophages

Four-day old head kidney primary macrophage cultures, prepared as above, were incubated with LPS (25 µg/mL), PolyI:C (50 µg/mL), and the pro-inflammatory cytokine rIFN-γ (20 ng/mL) [32].

After incubation for 4, 8 and 24 h the cells were resuspended in TRI Reagent® for RNA extraction and cDNA synthesis as described above.

2.5.3. Cell lines

After an overnight incubation post-passaging, the cell lines (RTGILL, RTG-2, RTL and RTS-11) were incubated with LPS, PolyI:C and rIFN-γ at the same concentrations used for the primary macrophage cultures or with medium alone for controls. Following incubation for 4, 8 and 24 h the cells were dissolved in TRI Reagent® and the RNA extracted.

2.6. Modulation of ROR-γ expression in vivo

2.6.1. Viral infection

To further investigate the regulation of trout ROR-γ upon viral exposure, the expression of this molecule was analysed in fish infected with the pathogenic viral hemorrhagic septicemia virus (VHSV), strain DK-F1. The challenge was performed as described by Campbell et al. [33]. Briefly, four fish were killed at 1, 2, 3, 4 and 6 days post-infection with DK-F1 (1×10^8 $TCID_{50}$/fish) or injection with control media as control. The head kidneys were collected for RNA extraction and cDNA synthesis. The expression level of ROR-γ was analysed by real-time PCR, as described above.

2.6.2. Vaccination and bacterial infection

Vaccination and bacterial challenge were performed as described by Harun et al. [34]. Briefly, trout were vaccinated with Aquavac™ against enteric redmouth disease (ERM) by intraperitoneal injection. The control group was handled in a similar way, but without vaccination. The fish were kept for 60 days in aerated freshwater tanks until they were challenged with a pathogenic strain (MT3072) of *Yersinia ruckeri* (0.5 mL per fish, 1×10^6 cfu/mL). Six fish were killed at 6 h, 1, 2 and 3 days post-infection and spleen and gill tissue was collected for total RNA extraction. The expression level of ROR-γ was analysed by real-time PCR, as described above.

2.7. Statistical analysis

Data were analysed statistically using the student *T*-test and one-way analysis of variance (ANOVA), with the LSD post hoc test used for comparison of means when appropriate, within the SPSS package 18.0 (SPSS Inc., Chicago). Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 Cloning and sequencing of ROR-γ

Two trout ROR-γ cDNA sequences, ROR-γa1 (EMBL accession number: **FM883712**) and ROR-γa2 (EMBL accession number: **FM883713**), were identified and shared a nucleotide similarity of 91.6% (**Supplementary Figure**). Both sequences contain a 5'-UTR of 303 bp and a coding region of 1,410 bp which translates into a 469 aa protein with predicted molecular mass of 52.8 and 52.9 kilodalton (kDa), respectively. In the 3'-UTR, two mRNA instability motifs (ATTTA) and three putative polyadenylation signal motifs (AATAAA) were also identified in both sequences. The second polyadenylation signal in the present transcripts is located 15 bp upstream of the poly A tail, suggesting that this is an authentic site.

Multiple alignment of the predicted amino acid sequences of vertebrate ROR-γ molecules revealed that the sequences are well conserved among different vertebrate species (**Figure 1A**). The presence of two highly conserved regions was identified in the trout sequences; a DNA-binding domain of 71 aa, which contains two C4 zinc-finger motifs, and a ligand-binding domain of 159 aa. Trout ROR-γ homologues showed a conservation of fourteen cysteine (Cys) residues amongst the vertebrate species selected, nine of them located in the DNA-binding domain. A high conservation of the AF-2 motif was also observed among all the ROR-γ molecules analysed, which in the trout and zebrafish homologues was characterised by the sequence LYREVF. In terms of homology, the trout proteins showed high amino acid similarity with zebrafish ROR-γ

homologues, with percentages of aa similarity >85%, followed by human and mouse ROR-γt (65.6 and 65.1%, respectively) and ROR-γ (63.1 and 62.8%, respectively). It was clear from the alignment that the N-terminal was more similar to ROR-γt than ROR-γ, and this was also born out by investigation of the genomic organisation of the human and zebrafish ROR-γ molecules. It was possible to detect a similar intron/exon organisation between human ROR-γt and the zebrafish homologues, with the presence of 10 exons, 8 of which were identical in terms of protein encoding nucleotides that included critically the 5'-end exons (**Figure 1B**). To further analyse the relationship of both trout sequences with other vertebrate molecules within the ROR family, a phylogenetic tree was constructed (**Figure 2**). The rainbow trout sequences clustered together with other known ROR-γ molecules forming a separate clade which was supported by a high bootstrap value of 97. Within the piscine cluster, they grouped more closely with zebrafish ROR-γa, suggesting that the nomenclature is correct, and hence why we term them ROR-γa1 and ROR-γa2.

3.2 Tissue distribution of trout ROR-γ

The expression of trout ROR-γ was examined in fourteen tissues from six healthy fish (**Figure 3**). Analysis revealed that this molecule was widely expressed, with the highest transcript level detected in muscle, followed by brain, heart and skin. This suggests a potential role of trout ROR-γ in these tissues. Its constitutive expression was also analysed in four established rainbow trout cell lines (RTGILL, RTG-2, RTL and RTS-11), as well as in primary head kidney leucocyte and macrophage cultures (**Figure 4**). In general, the trout cell lines had relatively high expression of this transcript, with the highest level being recorded in RTL, followed by RTGILL cells. In the primary cultures, interestingly, the expression of ROR-γ was higher in macrophages obtained from 4-day old head kidney primary cultures than in the mixed population of head kidney leucocytes. Since trout ROR-γa1/2 and zebrafish ROR-γa/b molecules are similar in length to mammalian ROR-γt (Fig. 1B), there is the possibility that transcripts with a longer N-terminus similar to

mammalian ROR-γ also occur in fish. Real-time PCR analysis performed above used primers designed against the coding region for robust detection of trout ROR-γ expression. Additional primers designed to the 50-end of the trout transcripts also amplified only a single product. Real-time PCR analysis using both primer pairs on the selected tissues (thymus, muscle, heart and brain) revealed a similar pattern of expression as seen in **Fig. 3** (data not shown). This suggests that the trout ROR-γ molecule analysed in this study is the major transcript in trout.

3.3 Modulation of trout ROR-γ in primary cultures

To analyse the modulation of trout ROR-γ, primary head kidney leucocytes obtained from four fish were incubated for 4, 8 or 24 h with a diverse range of stimulants including a T cell inducer (PHA), stimulators of intracellular signalling pathways (PMA and CI), an immunosuppressant (DM) and the bacterial pathogen associated molecular pattern (PAMP) LPS (**Figure 5**). ROR-γ was modulated negatively by PMA plus CI, CI, PHA or PMA, at different time points, with fold changes as low as 0.15. Incubation with LPS or DM had no effect on ROR-γ expression.

Since the constitutive expression of this molecule was higher in macrophages isolated from head kidney than in the mixed cell population obtained from the same tissue, head kidney macrophages were used in a further stimulation experiment. Four day old head kidney macrophages were incubated with LPS, with a synthetic analogue of double stranded RNA (PolyI:C), or with the pro-inflammatory cytokine rIFN-γ, known to be involved in T_H1 responses (**Figure 6A**). As observed previously, trout ROR-γ was mainly modulated negatively, in this case, by the presence of all stimulants used after a 4 and 8 h stimulation, with fold changes of 0.58 or below.

3.4 Modulation of trout ROR-γ in cell lines

After analysing the modulation of ROR-γ in primary cell cultures, the regulation of this molecule was also investigated using four established trout cell lines: RTGILL, RTG-2, RTL and RTS-11. The cells were also incubated with LPS, PolyI:C and rIFN-γ for 4, 8 and 24 h (**Figure 6B**). The presence of PolyI:C and rIFN-γ generally down-regulated the expression of ROR-γ (with some variation in kinetics) in all the cell lines studied. However, in contrast LPS was found to up-regulate ROR-γ expression in RTGILL, RTG-2 and RTL cells, although it down-regulated the expression in RTS-11 cells.

3.5 Modulation of trout ROR-γ in vivo

To assess the involvement of trout ROR-γ upon a viral infection and to investigate if it would also be negatively modulated, the expression of this gene was analysed in head kidney samples from four fish at several time points (days 1, 2, 3, 4 and 6) after a VSHV challenge (**Figure 7**). Trout ROR-γ expression was indeed down-regulated significantly ($P < 0.05$) at days 1, 2 and 3 post-infection, with fold changes of around 0.49, 0.18 and 0.26, respectively.

Since T_H17-type responses might be expected in fish vaccinated against extracellular bacteria, we also studied the expression of ROR-γ in trout that had been previously given a commercial vaccine against the Gram negative bacterial pathogen *Yersinia ruckeri* and then challenged 60 days later, with unvaccinated fish used as the control (**Figure 8**). In the spleen ROR-γ expression was significantly increased ($P < 0.05$) at days 1, 2 and 3 days post-infection in vaccinated fish *vs* the control unvaccinated fish, with ~8-fold increase apparent by day 3. In the gills there was a small down-regulation at 1 day post-infection in vaccinated fish *vs* unvaccinated fish, but a significant increase was again apparent at day 3, albeit lower than that seen in the spleen.

4. Discussion

This paper reports the cloning and characterisation of two ROR-γ homologues, ROR-γa1 and -γa2, in rainbow trout (*Oncorhynchus mykiss*). The two mRNA sequences consist of 2,713 and 2,801 nucleotides respectively, translating into a 469 aa protein in both cases. The trout ROR-γ molecules share a high homology of 98.3% and 99.6% identity and similarity, respectively. A multiple aa alignment of the different vertebrate ROR-γ molecules revealed a high conservation of DNA- and ligand-binding domains, which are characteristic features of the nuclear receptor family [3,9]. The present results also indicated that the AF-2 motif displayed a high conservation across vertebrates. This was not unexpected since this motif was reported to be highly conserved amongst all ROR family members [6-8]. In terms of homology with other vertebrate species, trout ROR-γ shared the highest similarity with zebrafish proteins (over 85%). A slightly higher homology to mammalian ROR-γt (over 65%) *vs* ROR-γ (62.8 - 63.1%) was also observed, and reflects the similarity of protein lengths between fish ROR-γ and ROR-γt. Thus, the first amino acids present in mammalian ROR-γ, which belong to the A/B domain, are absent in ROR-γt as well as in the piscine proteins. This fact was also apparent when comparing the genomic organisation of human and zebrafish ROR-γ molecules, with the zebrafish homologues lacking the presence of one exon as observed in the ROR-γt splice variant. A phylogenetic tree constructed using protein sequences belonging to the ROR family, demonstrated that both trout ROR-γ molecules closely grouped with the zebrafish homologues, and branched with other known vertebrate ROR-γ molecules, forming an independent clade separate to ROR-α and -β. Taken together, these results suggest that the trout ROR-γa1 and -γa2 are homologues of vertebrate ROR-γ, and are more similar to the mammalian splice variant ROR-γt. The cloning of two isotypes in rainbow trout was not surprising since the duplication of this gene has also been observed in another piscine species (zebrafish) [8]. However, the two zebrafish ROR-γ molecules did not group separately with the trout molecules, with ROR-γa apparently more closely related to both trout molecules, and hence the reason to call the trout molecules ROR-γa1 and ROR-γa2. The occurrence of the duplicated genes in trout is thus

most likely a result of the known ancestral genome duplication event that occurred in salmonids [35].

In the present study, tissue distribution analysis demonstrated that rainbow trout ROR-γ expression was highest in muscle followed by brain, heart and skin. This result is in agreement with previous mammalian studies, where ROR-γ is widely expressed in a variety of tissues, with the skeletal muscle having the highest transcript level, followed by the heart and brain [11-13,36]. In fish a weak expression of this gene in cardiac muscle and somites has been reported in zebrafish larvae, as well as in the brain [8]. The role of ROR-γ in these organs is still unclear, but it has been suggested that it is involved in the regulation of muscle metabolism and growth control [37]. When analysing the expression of ROR-γ in trout cell lines and primary cultures, it was found that this gene was highly expressed in the fibroblast-like cell lines. The use of mammalian fibroblast-like cell lines to study the regulation of ROR-γ has been reported [11,38], and suggests an important role of this molecule in this cell type. Further work is required to investigate the function of trout ROR-γ within the various tissues and cell lines where this gene is highly expressed.

In order to analyse if trout ROR-γ expression could be modulated in primary leucocyte cultures, head kidney leucocytes were incubated with a range of stimulants. It was observed that PHA had a negative effect on the expression of ROR-γ in contrast to the expression of other master transcription factors for regulatory T (FoxP3a and b), T_H1 (T-bet) and T_H2 (GATA-3) cell development which were induced by this mitogen in trout under comparable experimental conditions [39,40]. This might indicate a possible negative feedback mechanism by which these transcription factors, specific to distinct T_H subsets, are regulated. In the same experiment, exposure to other stimulants (PMA and CI) also resulted in the down-regulation of this molecule. Phorbol esters, such as PMA, activate protein kinase C, bypassing the classical signal transduction pathways. They exert a variety of effects in biological systems that include proliferation, tumour

promotion, cell differentiation and death [41,42,43]. It is possible that exposure to PMA led to cell death in the time frame used affecting the cell populations that express ROR-γ.

To further investigate the possible modulation of trout ROR-γ a sub-population of leucocytes (macrophages) was studied. Head kidney derived macrophages were incubated with the PAMPs PolyI:C and LPS, and also a pro-inflammatory cytokine rIFN-γ. All stimulants had a negative effect on ROR-γ expression. IFN-γ released from T_H1 cells has been reported to suppress T_H17 differentiation, possibly as a protective mechanism to regulate excessive inflammation [44,45]. Therefore, if trout ROR-γ is a potential T_H17 master regulator in fish, such an antagonising effect of IFN-γ on its expression would not be surprising. PolyI:C, a synthetic double stranded RNA which acts as a viral PAMP, is known to induce a T_H1-type response in fish [32]. Suppression of the transcript level of ROR-γ by this stimulant would also be understandable if ROR-γ is involved in inducing a T_H17-type response in fish, which evolved to eradicate extracellular pathogens [46,47]. T_H17-type responses have been described in fish [48] and IL-22 expression in gills appears to be a useful marker of vaccine-induced bacterial resistance [34,49].

Modulation of ROR-γ was further investigated by incubating four trout cell lines with rIFN-γ, PolyI:C and LPS. Overall, these stimulants triggered a very similar effect on ROR-γ expression in non-leucocytes (RTL, RTG-2 and RTGILL), with rIFN-γ and PolyI:C inducing a clear suppressive effect, as seen in macrophage primary cultures. However, in contrast to the negative effect of LPS in primary cultures, this stimulant was a good inducer of ROR-γ in these non-leucocyte cell lines. A similar pattern of modulation was observed when using the monocyte/macrophage cell line RTS-11, with the difference that LPS reduced the expression of ROR-γ as seen in head kidney macrophages. Clearly trout ROR-γ can be differentially modulated in different cell types. Mammalian studies have reported that activation of transcription of ROR family members (ROR-α and -β isotypes) is cell type dependent, which can potentially explain the different expression patterns seen when using distinct cell types but the same stimulus [9].

VHSV is an aquatic rhabdovirus, the aetiological agent of viral hemorrhagic septicaemia, which is responsible for causing losses of rainbow trout stocks in European aquaculture [50]. It causes haemorrhages in various tissues including internal organs, with the spleen and kidney being the most targeted by the virus in rainbow trout [50]. In this study a down-regulation of ROR-γ was seen over the first three days after viral infection. Again it can be hypothesized that T_H17 type responses may have a minor role in antiviral responses, where T_H1 cytokines would be expected to trigger protection against intracellular pathogens [17]. Thus, if trout have similar types of adaptive immunity this could potentially explain the suppression of ROR-γ upon exposure to a virus.

Yersinia ruckeri is the causative agent of enteric redmouth disease (ERM), affecting mainly salmonids, and can cause significant economic losses in the trout farming industry [51,52]. This pathogen is known to colonise vascularised tissues, including the spleen and head kidney [52]. Recent studies have reported that vaccine induced protection of rainbow trout to *Y. ruckeri* is correlated with a T_H17-type response in the gills where a high induction of IL-22 expression is seen shortly after bacterial challenge of the vaccinated fish [34]. It is important to note that unvaccinated infected fish show symptoms of the disease by day 3 post-infection and fish begin to die shortly afterwards. Samples from the study of Harun et al. [34] were used here to examine ROR-γ expression in the spleen and gills of vaccinated and unvaccinated fish post-infection. Interestingly ROR-γ expression was significantly up-regulated in both tissues in the vaccinated fish, at days 1 to 3 in the spleen and day 3 in the gills. These results are in line with the concept that trout ROR-γ could be involved in T_H17-type responses in fish, taking into account that a number of T_H17 cytokines have already been characterised in several teleost species [49,53,54]. However, it is also important to note that T_H17 cells are not the only cell type that can express ROR-γt and secrete IL-17 and/or IL-22. Recent studies have reported that a sub-population of mucosal NK cells and NKT cells also express these molecules [18,19,55].

In summary, this report describes the cloning and characterisation of two trout ROR-γ homologues for the first time in salmonids, and their modulation upon treatment with different stimuli. The high expression of this gene in muscle and brain, obtained from healthy fish, and in non-leucocyte cell lines, indicates a potential role of ROR-γ in these tissues/cell types. The expression of ROR-γ in these cells was induced by LPS but suppressed by PolyI:C and rIFN-γ. Similarly, in virus challenged fish ROR-γ expression was suppressed. However, in vaccinated fish that are subsequently challenged with the homologous bacteria the expression of ROR-γ was induced 1-3 days post-infection. Overall, the sequence and expression analysis lead us to speculate that trout ROR-γ could function in a similar way to mammalian ROR-γt, at least in terms of having a role in responses to extracellular microbial infections. In addition, since particular stimulants triggered distinct effects on ROR-γ expression according to the cell type studied, suggests that the regulatory mechanism(s) that govern the expression of this gene are most likely cell-type dependent.

5. Acknowledgments

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Figure legends

Figure 1A. Multiple alignment of known ROR-γ molecules. Conserved amino acids are highlighted in black, with the conserved Cys residues highlighted in grey. Arrows indicate predicted DNA- and ligand-binding domains, and the AF-2 region (ΦΦXE/DΦΦ) is boxed. The accession numbers of the ROR-γ proteins are as follow: zebrafish ROR-γa: **NP_001076288** and ROR-γb: **XP_690743**; human ROR-γ: **NP_005051** and ROR-γt: **NP_001001523**; mouse ROR-γ: **NP_035411** and ROR-γt: **CAA10661**. Note: Identical residues are identified as *****, whereas similar residues are identified as **.** . **Figure 1B.** Genomic organisation of human and zebrafish ROR-γ molecules. The coding region is represented as black boxes, the UTRs as white boxes and introns as grey lines.

Figure 2. Phylogenetic tree showing the relationship of both trout ROR-γ amino acid sequences with other known genes of the ROR family members. The amino acid sequences were aligned using CLUSTAL W and the tree constructed by the Neighbor-Joining (N-J) method supported with 1,000 bootstrap replications using MEGA 4.1 software. The accession numbers of the sequences used for the analysis are: **ROR-α**: human: **NP_599023**; cow: **NP_001179790**; mouse: **NP_038674**; chicken: **XP_413763**; xenopus: **NP_001072663**; zebrafish: **NP_001103637** and fugu **ENSTRUP00000026635**. **ROR-β**: zebrafish: **ABO15413**; chicken: **Q98934**; human: **BAH02286**; horse: **XP_001488198**; cow: **NP_001179587** and mouse: **NP_666207**. **ROR-γ**: zebrafish homologues a: **NP_001076288** and b: **XP_690743**; mouse: **NP_035411**; cow: **NP_001076920**; human: **NP_005051** and human homologue t: **NP_001001523**. Trout ROR-γ proteins are in bold and underlined.

Figure 3. Expression of trout ROR-γ in tissues (tail fins, head kidney, gonad, thymus, spleen, caudal kidney, scales, intestine, gills, liver, skin, heart, brain and muscle) from healthy fish, as detected by real-time PCR analysis. Data are averages + standard error (n=6) of ROR-γ expression after being normalised to that of elongation factor (EF)-1α.

Figure 4. Expression of trout ROR-γ in primary cultures and established cell lines as detected by real-time PCR analysis. Data are averages + standard error (n=4) of ROR-γ expression after normalisation to that of EF-1α. HK = head kidney leucocytes.

Figure 5. Expression of trout ROR-γ in head kidney primary cultures after incubation with the following stimulants: PMA (100 ng/mL) and CI (0.5 μg/mL), CI (0.5 μg/mL), PHA (10 μg/mL), PMA (100 ng/mL), DM (0.5 μg/mL), or LPS (25 μg/mL), Expression of ROR-γ was detected by real-time PCR analysis and normalised to the expression of EF-1α and controls, as per the Pfaffl method [31] and presented as fold change. Results are averages + standard error (n=4). Asterisks indicate significant differences ($P < 0.05$) relative to the control.

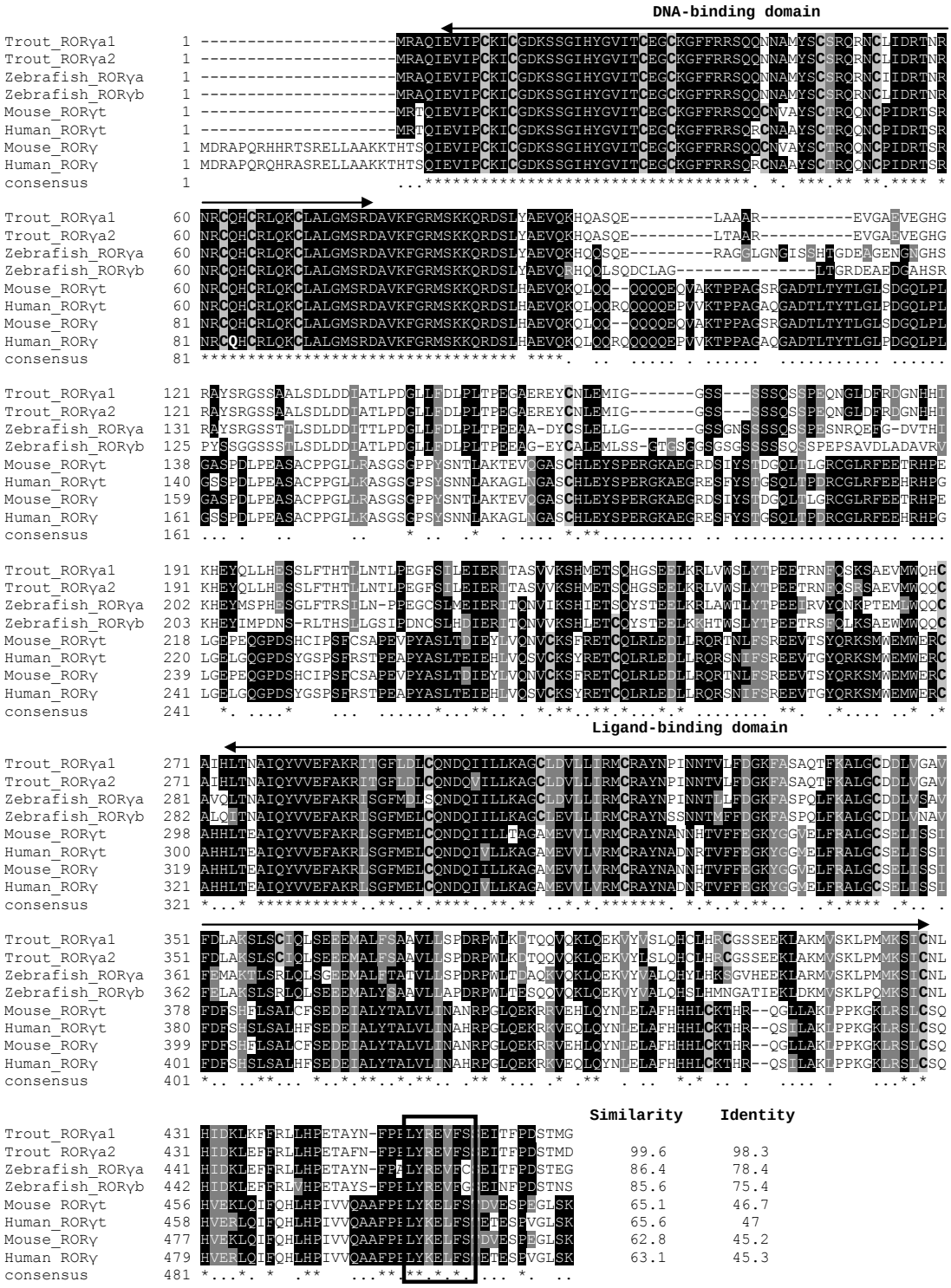
Figure 6. Expression of trout ROR-γ in 4-day old macrophage primary cultures (**A**) and cell lines (**B**) after incubation with PolyI:C (50 μg/ml), LPS (25 μg/ml) and rIFN-γ (25 ng/mL) for 4, 8 and 24 h. Expression was detected by real-time PCR analysis. Expression of ROR-γ was normalised to the expression of EF-1α and controls, as per the Pfaffl method [31] and presented as fold change. Results are averages + standard error (n=4). Asterisks indicate significant differences ($P < 0.05$) relative to the control.

Figure 7. Expression of trout ROR-γ during a viral infection. Rainbow trout were injected intraperitoneally with VHSV (strain DK-F1; 1 x 10^8 $TCID_{50}$/mL) or control media. Head kidney tissue was collected at days 1, 2, 3, 4 and 6 after challenge, and RNA extracted for real-time PCR analysis. Expression of ROR-γ was normalised to the expression of EF-1α and controls, as per the Pfaffl method [31] and presented as fold change. Results are averages + standard error (n=4). Asterisks indicate significant differences ($P < 0.05$) relative to the control.

Figure 8. Expression of trout ROR-γ following bacterial infection of vaccinated (60 days earlier with Aquavac™) and unvaccinated fish. Rainbow trout were injected intraperitoneally with *Yersinia ruckeri* (0.5 mL, 1x10^6 cfu/fish) and spleen and gill tissue collected at 6 h, 1, 2 and 3 days after challenge. RNA was extracted for real-time PCR analysis, and data were analysed using the Pfaffl method [31] and expressed as fold change relative to the unvaccinated fish. Results are averages + standard error (n=5), and asterisks indicate significant differences ($P < 0.05$) relative to the unvaccinated fish.

Figure 1.

A


DNA-binding domain
Ligand-binding domain
Trout_RORγa1
Trout_RORγa2
Zebrafish_RORγa
Zebrafish_RORγb
Mouse_RORγt
Human_RORγt
Mouse_RORγ
Human_RORγ
consensus
Similarity
Identity
Trout_RORγa2 99.6 98.3
Zebrafish_RORγa 86.4 78.4
Zebrafish_RORγb 85.6 75.4
Mouse_RORγt 65.1 46.7
Human_RORγt 65.6 47
Mouse_RORγ 62.8 45.2
Human_RORγ 63.1 45.3

B

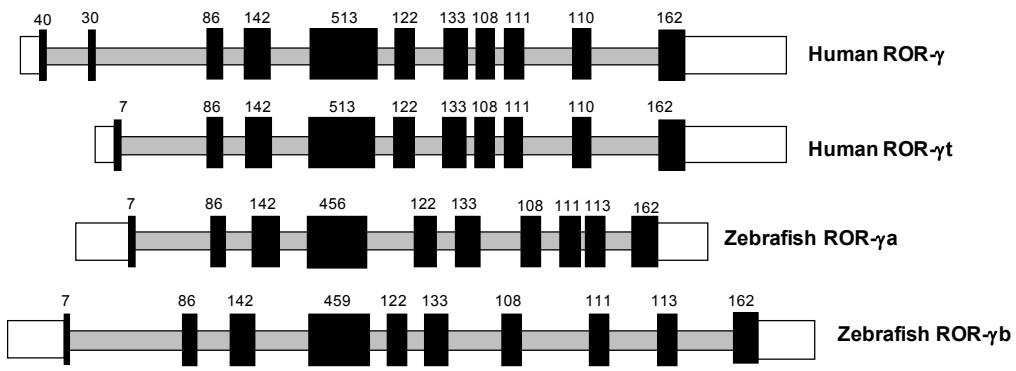

Human ROR-γ
Human ROR-γt
Zebrafish ROR-γa
Zebrafish ROR-γb

Figure 2.

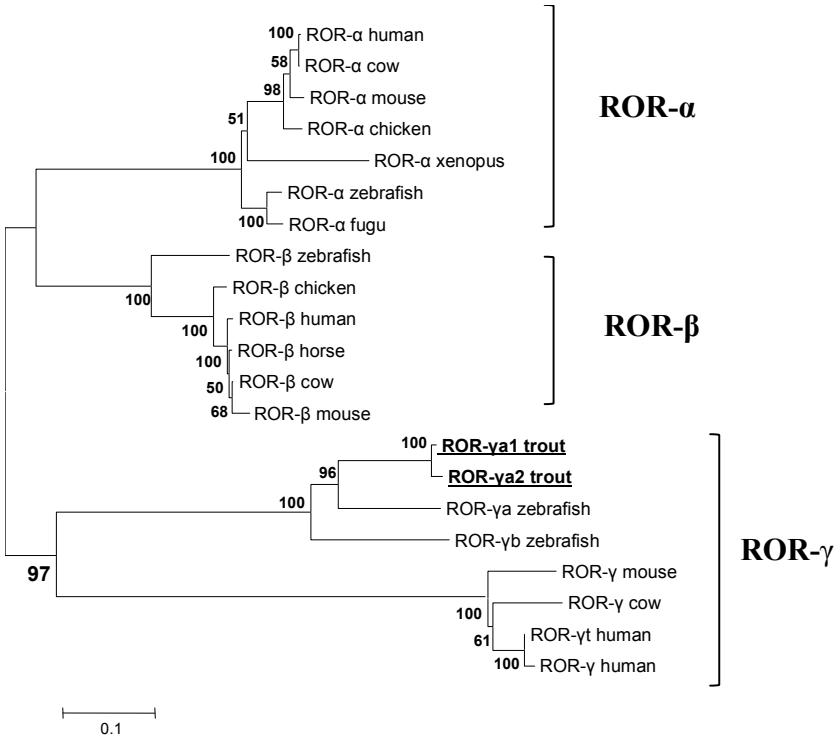

100
ROR-α human
58
ROR-α cow
98
ROR-α mouse
51
ROR-α chicken
100
ROR-α xenopus
ROR-α zebrafish
100
ROR-α fugu
ROR-α
ROR-β zebrafish
100
ROR-β chicken
100
ROR-β human
100
ROR-β horse
50
ROR-β cow
68
ROR-β mouse
ROR-β
100
ROR-γa1 trout
96
ROR-γa2 trout
100
ROR-γa zebrafish
ROR-γb zebrafish
97
ROR-γ mouse
100
ROR-γ cow
61
ROR-γt human
100
ROR-γ human
ROR-γ
0.1

Figure 3.

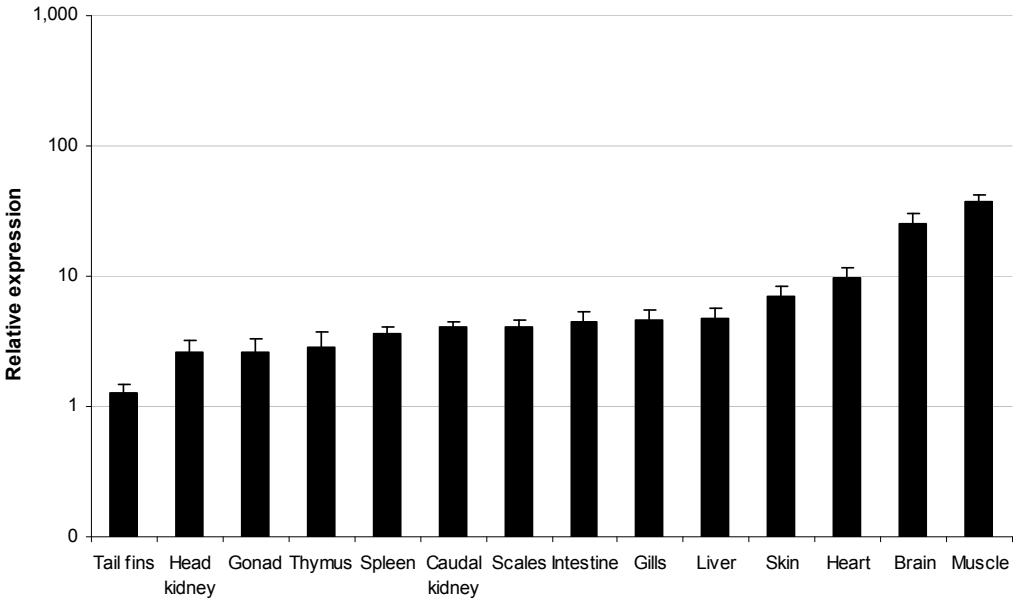

1,000
100
10
1
0
Relative expression
Tail fins
Head kidney
Gonad
Thymus
Spleen
Caudal kidney
Scales
Intestine
Gills
Liver
Skin
Heart
Brain
Muscle

Figure 4.

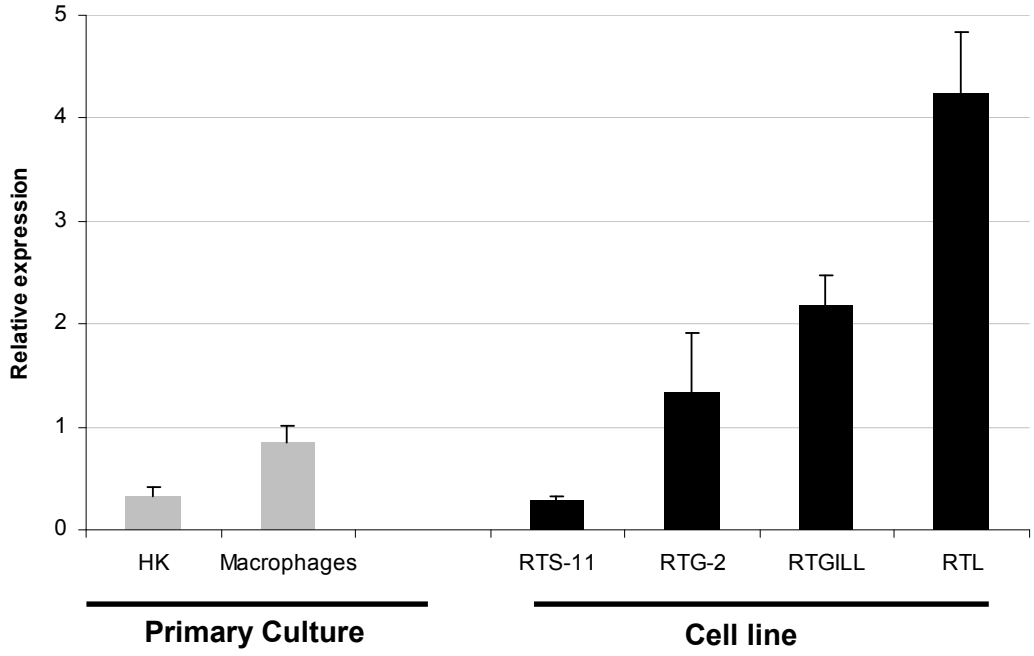

Relative expression
5
4
3
2
1
0
HK
Macrophages
RTS-11
RTG-2
RTGILL
RTL
Primary Culture
Cell line

Figure 5.

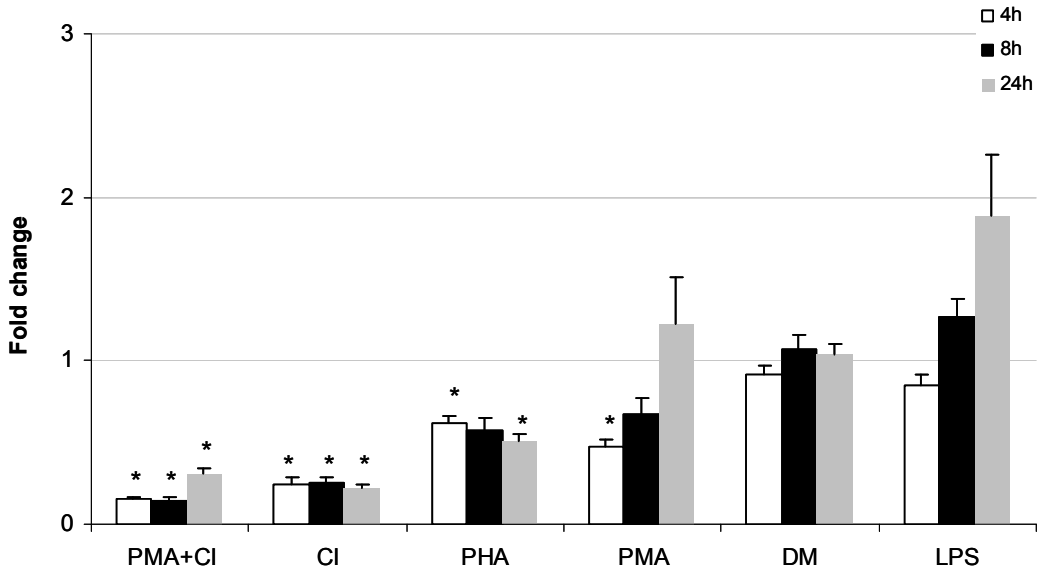
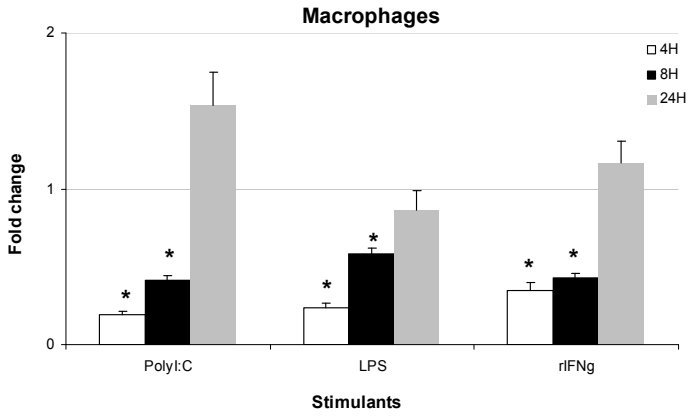


Figure 6.

A


Macrophages
4H
8H
24H
Fold change
2
1
0
PolyI:C
LPS
rIFNg
Stimulants

B

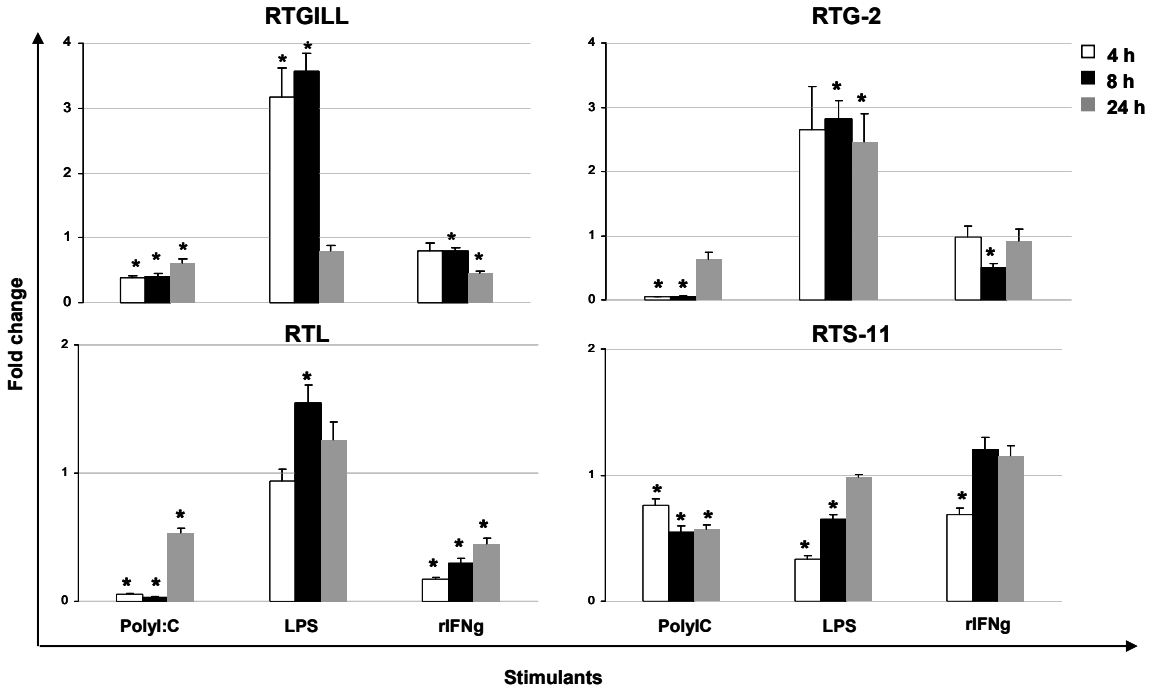

RTGILL
RTG-2
RTL
RTS-11
4 h
8 h
24 h
Fold change
PolyI:C
LPS
rIFNg
PolyIC
LPS
rIFNg
Stimulants

Figure 7.

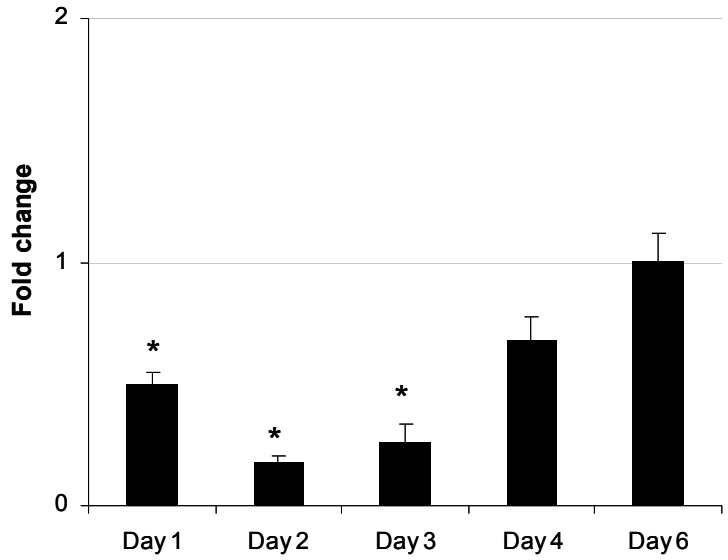


Figure 8.