Identification of IL-34 in teleost fish: differential expression of rainbow trout IL-34, MCSF1 and MCSF2, ligands of the MCSF receptor

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Abstract

The mononuclear phagocyte system is composed of monocytes, macrophages and dendritic cells and has crucial roles in inflammation, autoimmunity, infection, cancer, organ transplantation and in maintaining organismal homeostasis. Interleukin-34 (IL-34) and macrophage colony stimulating factor (MCSF), both signalling through the MCSF receptor, regulate the mononuclear phagocyte system. A single IL-34 and MCSF gene are present in tetrapods. Two types of MCSF exist in teleost fish which is resulted from teleost-wide whole genome duplication. In this report, we first identified and sequence analyzed six IL-34 genes in five teleost fish, rainbow trout, fugu, Atlantic salmon, catfish and zebrafish. The fish IL-34 molecules had a higher identity within fish group but low identities to IL-34s from birds (27.2-33.8%) and mammals (22.2-31.4%). However, they grouped with tetrapod IL-34 molecules in phylogenetic tree analysis, had a similar 7 exon/6 intron gene organisation, and genes in the IL-34 loci were syntenically conserved. In addition, the regions of the four main helices, along with a critical N-glycosylation site were well conserved. Taken together these data suggest that the teleost IL-34 genes described in this report are orthologues of tetrapod IL-34.

Comparative expression study of the three trout MCSFR ligands revealed that IL-34, MCSF1 and MCSF2 are differentially expressed in tissues and cell lines. The expression of MCSF1 and MCSF2 showed great variance in different tissues and cell lines, suggesting a role in the differentiation and maintenance of specific macrophage lineages in specific locations. The relatively high levels of IL-34 expression across different tissues suggests a homeostatic role of IL-34 for the macrophage lineage in fish. One striking observation in the present study was the lack of induction of MCSF1 and MCSF2 expression but the quick induction of IL-34 expression by PAMPs and inflammatory cytokines in cell lines and primary head kidney macrophages in rainbow trout. In a parasitic proliferative kidney disease (PKD) model, the expression of IL-34 but not the dominant MCSF2 was affected by PKD, suggesting an involvement of macrophage function in this disease model. Thus IL-34 expression is sensitive to inflammatory stimuli and may regulate macrophage biology once up-regulated.

Key words: teleost fish, IL-34, MCSF, MCSFR, gene cloning, differential expression

1. Introduction

The monocyte/macrophage lineage cells, including blood monocytes and resident tissue macrophages, are evolutionally conserved and the first line of defense against pathogens. They maintain homeostasis, and have trophic functions ranging from bone morphogenesis to neuronal patterning in sexual development and from angiogenesis to adipogenesis (Pollard, 2009; Wang et al., 2012). The development of monocytes and macrophages requires macrophage colony-stimulating factor (MCSF, also known as colony-stimulating factor 1, CSF1) through its binding to the MCSF receptor (MCSFR, also known as CSF1R or CD115) on bone marrow progenitor cells, which results in their proliferation and differentiation into monocytes and macrophages. All MCSF effects are mediated through MCSFR. Thus $Csf1r^{-/-}$ mice (mice deficient in MCSFR) show all the defects of $Csf1^{op/op}$ mice (mutant mice that lack MCSF), such as deficiency in most tissue macrophages (Dai et al., 20002). However, $Csf1r^{-/-}$ mice have more severe defects in several monocyte/macrophage subsets than $Csf1^{op/op}$ mice. For example, microglia (resident macrophages of the central nervous system) and Langerhans cells (resident dendritic cells of the epidermis) are absent from $Csf1r^{-/-}$ mice but present in $Csf1^{op/op}$ mice (Witmer-Pack et al., 1993). Such discrepancies suggest the existence of an alternative ligand for MCSFR that can partially compensate for the absence of MCSF in $Csf1^{op/op}$ mice. Indeed, interleukin 34 (IL-34), a molecule lacking discernible sequence similarity to MCSF, has been recently identified as the second functional ligand for MCSFR in mammals (Lin et al., 2008; Wei et al., 2010) and birds (Garceau et al., 2010).

Despite a lack of appreciable sequence similarity with other proteins, IL-34 was recognised as a short chain helical cytokine belonging to the same family as MCSF and stem cell factor (Garceau et al., 2010). Mammalian IL-34 has a distinctive antiparallel four-helix bundle cytokine core structure consisting of long aA, aB, aC, and aD helices as with MCSF (Liu et al., 2012; Ma et al., 2012). Outside this core portion, there are two additional shorter helices α1 and α2, connecting aA and aB, and aC and aD, respectively, that are packed against aB and aD, and associated with the 4 longer helices through continuous hydrophobic interactions. The human and mouse IL-34 is further locked by two intramolecular disulfide pairs located at the pole of each protomer that shares no structural similiarity with disulfide bonds in MCSF. The first disulfide bridge connects helices aA and aD, while the second locks the C-terminal extension to the end of

aD (Liu et al. 2012; Ma et al., 2012). Unlike MCSF, IL-34 is a non-covalently linked homodimer without an intermolecular disulfide bridge that is used to covalently cross-link the two monomers in the MCSF dimer (Liu et al., 2012). Furthermore, an N-glycosylation site is conserved in helix a1 in mammalian and bird IL-34 molecules. The glycan serves to fill the cavity between the helices α1 and αC and is critical for IL-34 stability in solution (Liu et al., 2012).

Through binding to MCSFR, IL-34 shares similar functions with MCSF. Thus, both cytokines support cell growth and survival in cell culture studies (Chihara et al., 2010; Misuno et al., 2011; Wei et al., 2010) and induce chemokines in human whole blood (Eda et al., 2010). IL-34 can also substitute for MCSF to support RANKL induced osteoclastogenesis (Baud'huin et al., 2010; Chen et al., 2011; Hwang et al., 2012; Nakamichi et al., 2012). The IL-34 gene, when expressed under the control of the MCSF promoter, can rescue the bone, osteoclast and tissue-macrophage defects of CSF-$1^{op/op}$ mice (Wei et al., 2010). However, the signal transduction mechanisms and biological activity of IL-34 and M-CSF are not identical. They differ in terms of the induction of chemokines and morphological changes induced in primary macrophages. IL-34 has been shown to induce a stronger but transient activation of MCSFR and downstream effectors and rapidly downregulates MCSFR expression (Chihara et al., 2010). Moreover, IL-34 and MCSF exhibit different spatiotemporal patterns of expression in both embryonic and adult tissues, which lead to complementary activation of MCSFR (Nandi et al., 2012; Wei et al., 2010). IL-34 but not MCSF mRNA is detected together with MCSFR in embryonic brain. Wang et al. (2012) found that the main sources of IL-34 are neurons in the brain and keratinocytes in the epidermis, and IL-34-deficient mice lack microglia and Langerhans cells. Thus, while IL-34 and MCSF resemble each other, they are not identical in their role in development, biological activity, and signal activation kinetics or strength.

IL-34 mRNA is broadly expressed in adult human tissues, including heart, brain, lung, liver, kidney, spleen, thymus, testis, ovary, small intestine, prostate and colon (Lin et al., 2008). Although the expression of both IL-34 and MCSF can be induced by proinflammatory cytokines, e.g. IL-1β and TNF-α, the signalling pathways involved in their induction are different. For example, MAPK is involved in the induction of IL-34 but not MCSF, by IL-1β and TNF-α (Eda et al., 2011). MCSF is expressed as a biologically active membrane spanning cell surface glycoprotein and can be cleaved to release a bioactive soluble MCSF (Pandit et al., 1992). In contrast, IL-34 is secreted. Whilst the cell surface isoforms of MCSF act locally in a cell-to-cell

contact manner, the secreted IL-34 and soluble MCSF can act at remote sites to induce a response.

MCSF has recently been identified in several fish species including rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*), with two isoforms of this cytokine (MCSF1, and MCSF2) identified in trout and zebrafish (Hanington et al., 2007; Wang et al., 2008). The two trout MCSF isoforms differ in their expression patterns in tissues and different cell populations. Recombinant fish MCSF was demonstrated to promote the proliferation of macrophages (Hanington et al., 2007; Wang et al., 2008), induce pro-inflammatory gene expression and enhance antimicrobial responses (Grayfer et al., 2009), suggesting a conservation of the monocyte/macrophage system and its regulation in vertebrates. In this report, we have identified and characterised IL-34 for the first time in fish, in rainbow trout, Atlantic salmon (*Salmo salar*), fugu (*Takifugu rubripes*), zebrafish, and catfish (*Ictalurus punctatus*). We have examined the expression of IL-34 together with the two other putative MCSFR ligands, MCSF1 and MCSF2, *in vivo* and *in vitro*, and found that the three ligands are differentially expressed and modulated by pathogen associated molecular patterns (PAMPs), proinflammatory cytokines and a parasitic infection.

2. Materials and methods

2.1. Identification and cloning of IL-34 in rainbow trout and fugu

Search of the expressed sequence tag (EST) database revealed two overlapping trout ESTs (Acc Nos. CA351788, CA368219) that when translated encode a peptide with homology to the N-termini of mammalian IL-34 molecules. The full-length sequence of the trout molecule was obtained by 3'-RACE using spleen SMART cDNA as described previously (Wang and Secombes, 2003). Primers (F1/F2, Table I) were designed in the 5'-untranslated region (UTR) and amplified a 1.5 kb product that contained the complete coding region and 3'-UTR.

The fugu IL-34 sequence was found by exploiting the conservation of synteny between the human and the fugu genomes. The fugu genome database was searched by basic local alignment search tool (BLAST) analysis (Altschul et al., 1990) using human metastasis suppressor 1-like (MTSS1L) and splicing factor 3b, subunit 3 (SF3B3) genes, the known neighbours of the human IL-34 gene. Two candidate fugu DNA scaffolds (764 and 2156) were obtained and a region encoding a possible fugu IL-34 homologue identified using various sequence analysis programs (Burge and Karlin, 1998). The prediction was confirmed by sequencing of a PCR product amplified by primers fIL34F1 and R1 (Table 1) from cDNA samples. The cDNA sequence was extended by 3'- and 5'-RACE using primers detailed in Table 1.

2.2. Sequence analysis of IL-34 homologues in other fish species

BLAST search at NCBI using the cloned trout and fugu IL-34 sequences identified tilapia and zebrafish IL-34 molecules predicted from the respective genomic sequence (Acc. Nos. I3JZ08 and B3DLJ8). Search of the EST database obtained multiple fish ESTs with ESTs from Atlantic salmon, catfish and zebrafish producing contigs of the complete coding region of IL-34 (details seen in Supplementary Figs. 2, 5 and 6). An additional salmon IL-34 gene (IL-34B) was also predicted from whole-genome shotgun contigs (WGS, Acc. No. AGKD01076819, detailed in Supplementary Fig. 3).

2.3. Gene organisation

The DNA sequences were retrieved from relevant databases. The gene organisation was determined by comparing the cDNA and genomic sequences using the online Spidey program

(http://www.ncbi.nlm.nih.gov/spidey) at NCBI.

2.4. Sequence analysis

The DNA sequences produced by cloning and the EST sequences retrieved from the database were assembled and analysed with the AlignIR program (LI-COR, Inc). The protein sequences were retrieved from the Expasy or NCBI protein databases. Global sequence comparisons were performed using the MatGAT program (V2.02, Campanella et al., 2003) using the scoring matrix BLOSUM60 with a gap open penalty of 10 and gap extension penalty of 1. Multiple sequence alignments were generated using ClustalW (Chenna et al., 2003) and shaded using BOXSHADE (version 3.21; www.ch.embnet.org/software/BOX_form.html). The signal peptide was predicted using SignalP 4.0 (Petersen et al., 2011). Finally phylogenetic trees were created by the neighbour-joining method using MEGA software (version 5.1, Tamura et al., 2011), and were bootstrapped 10,000 times.

2.5. Expression of IL-34, MCSF1 and MCSF2 in healthy trout and four trout cell lines

Six healthy rainbow trout (Mean±SEM =106.0±5.2 g) were killed and fourteen tissues (gills, thymus, scales, skin, muscle, liver, spleen, ovary, head kidney, caudal kidney, intestine, heart, tail fins and brain) were collected and homogenized in Trizol (Invitrogen). The RNA preparation and cDNA synthesis were as described previously (Wang et al., 2011a, b).

The primers (Table 1) for real-time-PCR analysis of gene expression were designed so that at least one primer crossed an intron and were pre-tested to ensure that each primer pair could not amplify genomic DNA using the real-time PCR protocols. The expression of trout IL-34, MCSF1 and MCSF2, as well as the house keeping gene elongation factor-1α (EF-1α) was quantified by real-time PCR as described previously (Wang et al., 2009, 2011a, b). For comparison, a standard was constructed using a mixture of equal mole amounts of purified PCR products amplified from cloned plasmids for each gene to be studied. A serial dilution of the standards was run along with the cDNA samples in the same 96-well PCR plate and served as a reference for quantification. The expression level of each gene was calculated as arbitrary units normalized to the expression of EF-1α. The expression level of MCSF1 in gills, the lowest level of all the ligands in all the tissues, was defined as 1.

The constitutive expression of the three ligands was also examined in four trout cell lines, RTS-11 (a mononuclear/macrophage-like cell line from spleen, Ganassin and Bols, 1998), RTL (an epithelial cell line from liver, Lee et al., 1993), RTG-2 (a fibroid cell lines from gonad, Wolf and Quimby, 1962) and RTGill (a fibroid cell line from gills, Schirmer et al., 1998). All cells were maintained in L-15 medium (Invitrogen) supplemented with 30% foetal calf serum (FCS, Labtech International, UK) for RTS-11 cells or 10% FCS for the other cell lines, and 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen). Total RNA was prepared from 1 day old cells after passage and real-time analysis of gene expression was as described above. The expression level of MCSF1 in RTGill, the lowest level of all the ligands in all the cell lines, was defined as 1.

2.6. Modulation of IL-34, MCSF1 and MCSF2 expression in four trout cell lines by PAMPs and IFN-γ

All cells were passaged 1 day before stimulation in 10% FCS at a concentration of 5-10x10^5 cells/ml. The cells were stimulated by direct addition of stimulants that were dissolved in L-15 medium. Three stimulants, *E. coli* LPS (25μg/ml, from strain 055:B5, Sigma), polyinosinic:polycytidylic acid (polyIC, 50 μg/ml, Sigma), recombinant trout IFN-γ (IFN-γ, 20 ng/ml, Wang et al., 2011) or medium alone as control, were used. The concentrations chosen for each stimulant were deemed optimal from previous studies (Wang et al., 2010; Holland et al., 2010). The treatments were terminated by dissolving the cells in TRIzol (Sigma) at 4 h, 8 h and 24 h post-stimulation and total RNA was prepared. Four replicates were used in each group. The real-time analysis of gene expression was as described in section 2.5. The fold changes were calculated as the average expression of the treatment groups divided by that of the time matched control group.

2.7. Modulation of IL-34 expression in primary head kidney macrophages

Primary head kidney (HK) macrophages were prepared from four trout as described previously (Costa et al., 2011). Four day old primary macrophages were then stimulated with a variety of PAMPs (LPS, 25 μg/ml; polyIC, 50 μg/ml), inflammatory cytokines (recombinant trout IL-1β, 20 ng/ml, Hong et al., 2001; IL-6, 200 ng/ml, Costa et al., 2011; IFN-γ, 20 ng/ml, Wang et al., 2011b), other immune stimulants (phorbol 12-myristate 13-acetate (PMA), 100 ng/ml; calcium ionophore (CI), 0.5 μg/ml; phytohaemagglutinin (PHA) from red kidney bean *Phaseolus vulgaris,* 10 μg/ml), as well as an immune suppressor (dexamethasone (DM), 0.5 μg/ml) for 4 h, 8 h and 24 h. All the chemicals were from Sigma-Aldrich and the stimulants were diluted in complete medium just before addition to the cells. The concentrations chosen for each stimulant

were deemed optimal from previous studies (Costa et al., 2011, Hong et al., 2001; Wang et al., 2009, 2011a, b). The treatments were terminated by dissolving the cells in TRIzol (Invitrogen). The real-time analysis of IL-34 expression was as described in section 2.5. The fold changes were calculated as the average expression of the treatment groups divided by that of the time matched control group. The expression of MCSF1 and MCSF2 was very low and not reported.

2.8. Modulation of IL-34, MCSF1 and MCSF2 expression by parasitic infection

Proliferative kidney disease (PKD) is a parasitic disease of salmonid fish caused by the myxozoan parasite *Tetracapsuloides bryosalmonae* (Bettge et al., 2009). The parasite infects the fish through the skin and gills and subsequently gains access to internal tissues, with the kidney being the main target organ. As the HK is the main organ of myeloid development in fish and primary HK macrophage increases IL-34 expression after stimulation (see results), the expression of IL-34, alongside MCSF1 and MCSF2, was analysed in HK of trout infected with *T. bryosalmonae* during a natural outbreak. Tissue collection and preparation of cDNA samples was as described previously (Wang et al., 2010). The severity of clinical pathology of each fish was analysed and a kidney swelling index assigned from 0 to 4, with 0 representing the control fish and 1 to 4 increasing pathology in the infected fish (Clifton-Hadley et al., 1987). The real-time PCR quantification of the genes was as described in section 2.5. The fold changes were calculated as the average expression of infected samples at each grade divided by that of the control group.

2.9. Statistical analysis

Real-time quantitative PCR measurements were analyzed using the SPSS package 20.0 (SPSS Inc. Chicago, Illinois). The arbitrary units for each sample were obtained after normalization to the lowest expression level in a data set that was defined as 1, and were log2 transformed to improve the normality of data distribution as described previously (Wang et al., 2011a). One-way ANOVA and the least significant difference (LSD) post hoc test were then used to analyze the expression data (Figs. 5B, 6 and 8), with $p \leq 0.05$ between groups considered significant. Since the expression data in Figs. 5A and 7 consisted of a set of samples from four individual fish, a Paired-Sample *T*-test was applied.

3. Results

3.1. Identification of teleost IL-34

The compiled trout IL-34 cDNA was 1464 bp with an open reading frame (ORF) of 615 bp encoding for 204 aa and a polyadenylation signal (AATAAA) 20 bp upstream of the poly A tail (Acc. No. FN820499, Supplementary Fig. 1). There are four upstream ATGs before the main ORF in the 5'-UTR and a mRNA instability motif (ATTTA) in the 3'-UTR. Using the trout IL-34 as bait, 28 salmon ESTs were obtained at NCBI that produced a contig of 1528 bp with an ORF of 615 bp encoding for salmon IL-34A (Supplementary Fig. 2). Similar to trout IL-34, the salmon IL-34A cDNA had 5 ATGs in the 5'-UTR, and an ATTTA motif and a polyadenylation signal in the 3'-UTR. A salmon WGS contig (NCBI ID: AGKD01076819) was further identified with a predicted ORF for 207 aa and was designated salmon IL-34B (Supplementary Fig. 3).

The extended cDNA sequence of fugu IL-34 contained a complete ORF of 681 bp encoding for 226 aa, with a predicted signal peptide at the N-terminus of the translation (Acc. No. AB691593, Supplementary Fig. 4). The 3'- and 5'-RACE products probably did not contain the full-length cDNA sequence, in that there were no upstream ATGs in the 5' UTR sequence and no polyadenylation site in the 3' UTR sequence.

Eight catfish ESTs were retrieved at NCBI, that made a contiguous sequence of 1691 bp encoding an ORF of 636 bp that translated into the catfish IL-34 protein of 211 aa (Supplementary Fig. 5). Two ATGs in the 5'-UTR, and an ATTTA motif and a polyadenylation signal in 3'-UTR were also observed in this catfish IL-34 cDNA. The ORF for zebrafish IL-34 was predicted from the genome sequence but no cDNA sequence had been reported previously. Six zebrafish ESTs were obtained at NCBI that gave a contiguous sequence of 1539 bp, with an ORF of 642 bp encoding the predicted protein of 213 aa (Acc. No. B3DLJ8). Two ATGs in the 5'-UTR and an ATTTA motif in the 3'-UTR were also present in this cDNA sequence (Supplementary Fig. 6).

The identities of teleost IL-34 were further confirmed by synteny analysis in human, chicken, zebrafish and fugu (Fig. 1). The human, chicken and zebrafish IL-34 genes are on Chromosome (Chr) 16, 11 and 18, respectively. The fugu IL-34 was on scaffold 764 and 2156 in the fugu genome (Ensembl release 68). The

genes, VAC14, COG4, MTSS1L, SF3B3, N4PB1, SIAH1 and LONP2, are linked to IL-34 on human Chr 16 and chicken Chr 11. The last five genes are also present in the zebrafish IL-34 locus on Chr 18, and the last four genes conserved in the fugu IL-34 locus (Fig. 1), suggesting that the teleost IL-34 genes are indeed orthologues of tetrapod IL-34.

3.2. Chracterisation of teleost IL-34

The teleost IL-34 aa sequences are sumarised in Table 2. They are 204-226 aa long with basic theoretical isoelectric points (pI) (8.36-9.42) except fugu IL-34 (pI=6.18). As with bird and mammalian IL-34 molecules, the teleost IL-34 molecules have a signal peptide at the N-terminal suggesting they are secreted. Two to six potential N-glycosylation sites are also present in each fish molecule (Table 2).

The trout IL-34 and salmon IL-34A share 95.1% identity to each other but only share 82.2% identity to salmon IL-34B (Table 3). All the fish IL-34 molecules from different families share comparable high identities (37.4-49.3%) except the fugu and tilapia IL-34s that share 63.6% identity to each other. However, the fish IL-34 molecules only share low identities to IL-34s from birds (27.2-33.8%) and mammals (22.2-31.4%). Identities within birds (76.7%) and mammals (67.8-71.9%) are relatively high (Table 3).

To further reveal the conservation and identity of fish IL-34 molecules, a multiple alignment was constructed (Fig. 2A). The human and mouse IL-34 have four long alpha helices (aA-D) that form the core “up-up-down-down” structure, in addition to two short helices (a1-2) (Liu et al., 2012; Ma et al., 2012). The four long helices, as well as the a1 helix are well conserved (Fig. 2A). The N-glycosylation site in helix a1, shown to be critical for IL-34 stability in solution (Liu et al., 2012), is also conserved in all vertebrates. An additional N-glycosylation site between helices aA and a1 is present in teleost species except tilapia (Fig. 2A). There are six conserved cysteine residues (C1, 2, 4, 5, 6 and 7 in the alignment) in mammalian IL-34, and four of them form two pairs of intramolecular disulfide bonds (C1-C6 and C4-C7, Ma et al., 2012) (Fig. 2B). Four of the six cysteine residues (C1, 2, 6 and 7) were also conserved in teleost IL-34 molecules, in addition to a fish specific cysteine residue C3. Thus the the teleost IL-34 may also have two intramolecular disulfide bonds (C1-C6 and C3-C7; Fig. 2B). However, the bird IL-34 molecules only have three conserved cysteine residues (C1, 2 and 6) and miss the final cysteine C7 present in both mammals and teleosts. Interestingly, C2 is conserved across all vertebrates but is reported to have no role in disulfide bond formation in mammalian

IL-34 (Ma et al., 2012). One noticable difference between mammals and other species (fish and birds) is the longer C-terminal tail in mammalian IL-34 (although fugu IL-34 is relatively long for fish), which is Pro-Ser-Thr-rich, a feature typical of flexible mucin-like O-linked glycosylation-rich sequences, that is missing in fish and bird IL-34 (Liu et al., 2012, Fig. 2A). The shorter C-terminal of teleost IL-34 contains many basic residues (K and R).

A phylogenetic tree was also constructed using mammalian MCSF as an outgroup (Fig. 3). All the IL-34 molecules from fish, birds and mammals grouped together with high bootstrap support (100%) and separate from MCSF, indicating that they are indeed orthologues. In agreement with the multiple alignments, the IL-34 molecules from teleost fish, birds and mammals form independent clades, a reflection of selection pressures in each vertbrate group.

It appeared that the IL-34 genes across vertebrates had a general 7 exon/6 intron structure, with one intron in the 5'-UTR. The first and last introns in the coding region are phase 1 and the rest are phase 0. One exception was fugu IL-34 that may have an 8 exon/7 intron structure resulting from an intron insertion in exon 5 (Fig. 4). Despite the conservation of exon number, exon size showed group specific features except in exons 4 and 5. Mammalian IL-34 genes had a large untranslated region in the first coding exon (exon 2) and a large coding region in the last exon compared to fish and bird IL-34 genes. In the chicken IL-34 gene exon 3 was similar in size to teleost IL-34 genes but exon 6 was more similar to mammalian IL-34 genes (Fig. 4).

3.3. Differential expression of IL-34, MCSF1 and MCSF2 *in vivo* and in cell lines

The expression of the three trout MCSFR ligands, IL-34, MCSF1 and MCSF2, was comparatively examined in fourteen tissues from six healthy trout, by real-time PCR (Fig. 5A). The expression of MCSF1 showed great variance between tissues, with the lowest level detected in the gills (arbitrary unit 1) and highest level in spleen (40,088). The expression of MCSF2 was also varied, with the highest expression level in the head kidney (2,529) and lowest in ovary (3). IL-34 was relatively highly expressed and less varied across tissues, with the highest level in gills (1,416) and lowest in ovary (57, Fig. 5A). The expression levels of IL-34 and MCSF2 differed significantly in all tissues, whilst IL-34 and MCSF1 differed in most tissues except in brain and tail fins (Fig. 5C). The expression levels of MCSF1 and MCSF2 were also different in most tissues, except liver, skin, thymus and heart.

Consistent with the *in vivo* expression, MCSF1 expression also showed greatest difference in cell lines with the lowest level (arbitrary unit 1) in RTGill (a cell line derived from gills) and highest level (53,696) in RTL (a cell line derived from liver) (Fig. 5B). MCSF2 expression was less varied, with the lowest expression level (2) in RTS-11 cells and the highest level (660) in RTGill cells. IL-34 expression was again relatively high and showed the least variation, with the highest level (3,045) in RTS-11 cells and lowest (592) in RTG-2 cells (Fig. 5B). The expression levels of the three ligands differed from each other in all the cell lines (Fig. 5C).

3.4. Differential modulation of IL-34, MCSF1 and MCSF2 in four trout cell lines

Trout MCSF1 expression was not increased in cell lines after 4 h of stimulation with PAMPs (LPS and polyIC) (Wang et al., 2008). To investigate if these PAMPs can modulate the other MCSFR ligands, four trout cell lines were stimulated with the same PAMPs, as well as IFN-γ, a known modulator of MCSF expression in mammals (Ogawa et al., 1994), for 4 h, 8 h and 24 h. The expression of both MCSF1 and MCSF2 was not increased by any of the stimulants at any of the time points except for MCSF1 expression in RTL and RTGill cells that was increased by polyIC at 24 h (Fig. 6). However, the expression of both genes was inhibited by these stimulants in a cell line- and time-dependent manner. In contrast, the expression of IL-34 was induced by all the three stimulants in all the four cell lines tested (Fig. 6). The highest induction (23-fold) of IL-34 expression like in RTS-11 cells was by LPS, whilst polyIC gave the largest increases in the other cell lines, e.g. RTL 26.7-fold, RTG-2 18.7-fold and RTGill 12-fold. A modest induction (less than 5-fold) of IL-34 expression was also seen in all cell lines after IFN-γ stimulation (Fig. 6).

3.5. Differential modulation of IL-34, MCSF1 and MCSF2 in adherent primary head kidney macrophages

As macrophages are a first line of defence *in vivo* and the macrophage like cell line RTS-11 expressed the highest level of IL-34 of the three MCSFR ligands (Fig. 5B), IL-34 expression was further examined in primary HK macrophages stimulated with PAMPs, proinflammatotory cytokines (recombinant trout IFN-γ, IL-1β and IL-6), other stimulants (PHA, PMA and CI) and an immuno-suppressant (DM). The expression of MCSF1 and MCSF2 was very low and showed no significant induction in these primary macrophages (data not shown). IL-34 expression was highly induced by LPS (up to 45.3 fold), polyIC (up to 36.9 fold), IFN-γ (up to 16.8 fold), IL-1β (up to 58.7 fold) and PHA (up to 4.4 fold), but was refractory to stimulation by IL-6,

CI and DM, and inhibited by PMA at 4 h and 8 h (Fig. 7).

3.6. Differential modulation of IL-34, MCSF1 and MCSF2 in head kidney by PKD

The kidney of teleost fish is a major lymphoid organ and a site of hematopoiesis and macrophage development (Zapata et al., 2006). PKD infection can lead to a massive granulomatous infiltration and proliferation of the interstitial tissue of the kidney (Holland et al., 2003). To investigate the potential involvement of macrophages in the disease, the expression of the three MCSFR ligands was examined in the kidney during a natural infection. The control fish were from the same source but not exposed to infection and parasite-infected fish were assigned a kidney swelling index from Grade 1 to 3. A modest increase (1.7-2.6 fold) of IL-34 expression was seen in all the infected fish (Fig. 8). However, the expression of MCSF2 was refractory to the disease and MCSF1 was decreased in advanced stages of the disease (over Grade 1).

4. Discussion

We report for the first time the identification and characterisation of IL-34 in teleost fish. IL-34 is another potential ligand for MCSFR, in addition to the other two potential ligands in teleosts, MCSF1 and MCSF2. The expression of IL-34 has been studied in rainbow trout and compared to the expression of MCSF1 and MCSF2. We found that the three ligands were differentially expressed *in vivo* and in cell lines, and that IL-34 was the main responder of the three ligands to inflammatory stimulation and parasite infection.

4.1. The teleost IL-34 gene

By cloning and database mining, we identified six IL-34 genes in five teleost fish, rainbow trout, fugu, Atlantic salmon, catfish and zebrafish. The fish IL-34 molecules had low identities to IL-34s from birds (27.2-33.8%) and mammals (22.2-31.4%) (Table 3). However, they grouped with tetrapod IL-34 molecules in phylogenetic tree analysis (Fig.3), had a similar 7 exon/6 intron gene organisation (Fig. 4), and genes in the IL-34 loci were syntenically conserved (Fig. 1). In addition, the regions of the four main helices, along with a critical N-glycosylation site were well conserved (Fig 2). Taken together these data suggest that the teleost IL-34 genes described in this report are orthologues of tetrapod IL-34.

Despite the above conserved features, the IL-34 molecules from fish, birds and mammals showed particular characteristics in each group. The mammalian IL-34s have a longer C-terminal tail that extends beyond the core four helix structure and that is P-S-T-rich. The C-terminal of teleost IL-34s are shorter (except fugu IL-34) and basic residue (K-R) rich. The bird IL-34s are the shortest and lack the last cysteine residue that is conserved in both fish and mammals. Interestingly, the C-terminal of mammalian IL-34 is not necessary for its function. Thus human recombinant IL-34 lacking the last 49 aa is as active as MCSF and slightly more active than full-length recombinant IL-34 in its ability to promote human monocyte viability (Ma et al., 2012). The Pro-Ser-Thr-rich tail in mammalian IL-34 has the potential for O-linked glycosylation (Liu et al., 2012) that may affect receptor binding or stability. Due to the K-R rich C-terminal, the fish IL-34s are basic except for fugu IL-34 which is acidic as a result of the extended D-E rich C-terminal. These differences in the C-terminal tail in fish and mammals may have an as yet unrecognised role in IL-34 biology.

The other major difference between the molecules is the potential to form disulfide bonds. Mammalian IL-34

has six conserved cysteine residues, whilst fish have five and birds only three. All the three cysteine residues in bird IL-34 are conserved in fish and mammals and form one putative conserved disulfide bond. Mammalian IL-34, and likely the fish IL-34, have one additional disulfide bond (Fig. 2). The potential of the remaining two cysteine residues of mammalian IL-34 to form a disulfide bond is unclear (Ma et al., 2012). Interestingly, one of the cysteine residues is well conserved in both fish and birds. The MCSF homodimer but not mammalian IL-34 is linked by an intermolecular disulfide bond. The role of this conserved cysteine residue in IL-34 remains to be determined.

Also, whilst the IL-34 genes across vertebrates have a general 7 exon/6 intron structure, the exon sizes are quite variable, with fish and mammals having the largest differences and birds somewhat in-between these two. Interestingly in fugu IL-34 an extra exon was present, the result of an intron insertion in the common exon 5. In addition, the coding region of the last exon of fugu IL-34 is longer than equivalent exons in other fish and bird IL-34 genes. A similar gene organisation has been found in *Tetraodon nigroviridis* IL-34 in the genome database (data not shown), suggesting these differences are lineage specific.

Due to the teleost fish wide whole genome duplication (FWGD) event (Meyer and Van de Peer, 2005), teleost fish possess two types of macrophage colony stimulating factors, e.g. trout MCSF1 and MCSF2 (Wang et al., 2008). We were only able to identify a single IL-34 in each fish species examined except in Atlantic salmon. However, the two salmon IL-34 genes share 82.2% identity at the protein level and in phylogenetic tree analysis they group closely together, suggesting they may have resulted from a further genome duplication event known to have happened in the ancestor of salmonids (Koop et al., 2008) although a tandem gene duplication event cannot be excluded at the momment.

4.2. Features of the teleost IL-34 transcript

Two to five ATG codons can be found in the 5'-UTR of the main ORF of IL-34 genes in trout, salmon, zebrafish and catfish, where full-length cDNA sequences are available (supplementary Figs. 1-3, 5-6). The chicken, human and mouse IL-34 genes also have multiple ATGs before the main ORF. These upstream ATGs are found within many eukaryotic transcripts and are known to regulate protein translation (Wethma et al., 2010). Thus, IL-34 gene expression across vertebrates may be controlled at the translational level by these upstream ATGs. Another common feature of IL-34 cDNA sequences is the presence of the ATTTA

motif in the 3'-UTR of fish, birds and mammals. The ATTTA motif in 3'-UTRs is implicated in the regulation of mRNA stability (Wu and Brewer, 2012; Roca et al., 2007), and indicates that IL-34 gene expression may be regulated at the mRNA and translational levels in addition to the transcriptional level.

4.3. The expression of three MCSFR ligands in tissues and cell lines

The expression patterns of mammalian IL-34 mRNA and MCSF mRNA are spatially and temporally distinct, which suggests that they have complementary rather than redundant roles in MCSFR activation *in vivo* (Wang et al., 2012). Due to the additional teleost-wide whole genome duplication, teleost fish possess two MCSF genes, as well as two MCSF receptors (unpublished results). Thus teleost fish have at least three ligands and two MCSFRs, and in salmonids there may be double this amount of ligands and receptors because of the additional WGD in the ancestor of salmonids, suggesting a complex regulation of monocyte/macrophage development in teleost fish.

In general, the three trout MCSFR ligands are differentially expressed in tissues and cell lines. The expression of MCSF1 and MCSF2, the membrane bound ligands of MCSFR, showed great variance in different tissues and cell lines, perhaps suggesting that these ligands have a role in the differentiation and maintenance of specific macrophage lineages in specific locations. Macrophage lineage cells populate every tissues of vertebrates with crucial functions in maintaining the homeostasis of many tissues as well as promoting inflammatory and repair responses to microbial, chemical and physical insults (Geissmann et al., 2010). The relatively high levels of expression of IL-34 across different tissues suggests a homeostatic role of IL-34 for the macrophage lineage in fish. Nevertheless, one striking observation in the present study was the lack of induction of MCSF1 and MCSF2 expression but the quick induction of IL-34 expression by PAMPs and inflammatory cytokines in cell lines and primary HK macrophages (Figs. 6-7). IL-34 expression was increased by LPS, polyIC, IL-1β and IFN-γ stimulation, and induction was quick, and in most cells peaked at 4 h after stimulation (the earliest time point examined). Thus IL-34 expression is sensitive to inflammatory stimuli and may regulate macrophage biology once up-regulated.

Macrophages and dendritic cells function as antigen presenting cells, and are critical for modulation of the T cell immune response (Almolda et al., 2011). The gills expressed the highest level of IL-34 amongst the fourteen tissues examined, and both gills and thymus expressed IL-34 at a level that was more than one order

higher than that of MCSF1 and MCSF2. The thymus is a site of T cell development in fish as in other vertebrates, and the gills have been shown recently to possess a unique T cell rich lymphoid tissue (Haugarvoll et al., 2008). Therefore, this expression pattern may suggest a role of IL-34, through its action on macrophage lineage, in fish T cell development.

4.4. The expression of IL-34 during proliferative kidney disease (PKD)

PKD is a parasitic disease of salmonid fish caused by the myxozoan parasite *T. bryosalmonae*. Fish are infected by parasite spores released from bryozoans which are the invertebrate host of the parasite. *T. bryosalmonae* infects the fish through skin and gills and afterwards invades inner organs, with the kidney being the main target organ where massive granulomatous infiltration and proliferation of the interstitial tissue occur post-infection (Bettge et al., 2009). The role of kidney macrophages in PKD is unclear. Previously we have shown that MCSF2 is the dominant isoform in the head kidney (Wang et al., 2008), but in this study its expression in head kidney was not affected by PKD. IL-34 is also highly expressed in head kidney and its induction by PKD at this site suggests an involvement of macrophage function in this disease model.

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Figure legends:

Fig. 1. Diagram to show gene synteny at the IL-34 loci in fugu, zebrafish, chicken and humans. The arrows indicate the transcriptional direction.

Fig. 2. Multiple alignment of teleost IL-34 amino acid sequences with selected tetrapod IL-34 molecules (A) and schematic diagram to show the secondary structure and potential intramolecular disulfide bonds in IL-34 (B). The multiple alignment was produced using ClustalW, and conserved amino acids shaded using BOXSHADE (version 3.21). The signal peptide, four long helices (aA-aD) that form the core four helical bundle and two additional short helices (a1 and a2) are indicated. The conserved cysteine residues (C1-C7) are indicated above the alignment and conserved N-glycosylation sites by a star. The accession numbers for sequences used in this alignment are given in Fig. 3.

Fig. 3. An unrooted phylogenetic tree of teleost IL-34 and selected tetrapod IL-34 molecules. The tree was constructed using amino acid multiple alignments and the neighbour-joining method within the MEGA5 program (Tamura et al., 2011). Node values represent percent bootstrap confidence derived from 10,000 replicates. The evolutionary distances were computed using the JTT matrix-based method. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Selected mammalian MCSF molecules were chosen as an outgroup. The accession number for each sequence is given after the species name and molecular type except for the fish IL-34 molecules from trout, salmon, fugu and catfish analysed in this report.

Fig. 4. Gene organisation of teleost and tetrapod IL-34 molecules. The gene organisation was predicted using the Spidey program. The grey and white boxes represent amino acid coding regions and untranslated regions within exons, respectively, and the black bars represent introns. The sizes (bp) of exons are numbered in the boxes and the intron phase is indicated under the bar. The fish IL-34 cDNA sequences are described in Supplementary Figs. 2 (salmon IL-34A), 4 (fugu), and 6 (zebrafish); and the genomic sequences are from WGS contigs AGKD01091083 and AGKD01005291 (salmon IL-34A); AGKD01076819 (salmon IL-34B); CABZ01008753 and CABZ01008752 (zebrafish IL-34), and fugu genome scaffolds 764 and 2156. The human, mouse and chicken IL-34 gene organisations were derived from AC020763 (DNA) and

NM_152456 (mRNA), AC139245 (DNA) and NM_001135100 (mRNA), and AADN03006282 (DNA) and XM_003641892 (mRNA), respectively.

Fig. 5. Differential expression patterns of trout IL-34, MCSF1 and MCSF2 transcripts in tissues and cell lines. The expression of trout IL-34, MCSF1 and MCSF2 in 14 tissues from healthy fish (A) and four cell lines (B) was determined by real-time PCR. The transcript level was first calculated using a serial dilution of references in the same run. The relative expression level was then expressed as arbitrary units normalized against the expression level of EF-1α. The expression levels of MCSF1 in gills (A) and RTGill (B), the lowest amongst the same data set, were defined as 1. The results represent the mean + SEM of six fish (A) and four flasks of cells (B). The ratios of the expression levels between trout IL-34, MCSF1 and MCSF2 and the p values comparing the difference are also shown (C). A paired sample t-test was applied to the tissue samples and one-way analysis of variance was used for the cell lines. The p value numbers shaded indicate a significant difference of the expression levels of the genes concerned.

Fig. 6. Modulation of expression of IL-34, MCSF1 and MCSF2 in four trout cell lines by PAMPs and IFN-γ. One day after passage, four cell lines, RTGill, RTL, RTG-2 and RTS-11, were stimulated with polyIC (50 μg/ml), LPS (25 μg/ml), and recombinant IFN-γ (20 ng/ml), or with medium only as control for 4 h, 8 h and 24 h and terminated by dissolving the cells in TRI reagent. The expression of trout IL-34, MCSF1 and MCSF2 was examined as in Fig. 5. A fold change that was calculated as the average expression level of stimulated samples divided by that of the time-matched controls is presented. The results represent the average + SEM from four flasks of cells. The relative significance of an LSD post hoc test after a significant one-way analysis of variance between the stimulated samples and control at the same time point is shown above the bars as: *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Fig. 7. Modulation of expression of trout IL-34, MCSF1 and MCSF2 in primary HK macrophages. Four day old HK primary macrophages were stimulated with LPS (25 μg/ml), polyIC (50 μg/ml), and recombinant IFN-γ (20 ng/ml), IL-1β (20 ng/ml), IL-6 (100 ng/ml), PHA (10 μg/ml), PMA (100 ng/ml), calcium ionophore (CI, 500 ng/ml) and dexamethasone (DM, 500 ng/ml) for 4 h, 8 h and 24 h. The RNA preparation and quantification of gene expression was as described in Fig. 5. Gene expression was expressed as a fold change that was calculated as the average expression level of stimulated samples divided by that of

the time-matched controls. The mean+SEM of four fish is shown. The p-values of a paired samples T test between stimulated samples and their time matched controls is shown above the bars as: *p≤0.05, **p≤0.01 and ***p≤0.001.

Fig. 8. Expression analysis of trout IL-34, MCSF1 and MCSF2 during a parasitic infection. Kidneys from rainbow trout infected with *T. bryosalmonae* or from unexposed fish (control) were collected during a natural infection. RNA was extracted and quantification of gene expression was as described in Fig. 5. The gene expression was expressed as a fold change that was calculated as the average expression level of each grade divided by that of the uninfected controls. Results are averages + standard error. The fish number was 11, 5, 9, 10 and 9 for control, Grade 1, 1-2, 2 and 3, respectively. The relative significance of an LSD post hoc test after a significant one-way analysis of variance between the infected and control samples is shown above the bars as: *p≤0.05 and ***p≤0.001.

Highlights

- The IL-34 gene has been identified in teleost fish for the first time.
- The IL-34 loci are syntenically conserved in fish, birds and mammals.
- The IL-34 gene has a general seven exon/six intron organisation across vertebrates.
- Fish IL-34 has a short, basic amino acid-rich C-terminal tail.
- IL-34 expression is induced by LPS, polyIC, IL-1β, IFN-γ, PHA and parasitic infection.

Table 1. Primers used for PCR cloning and real-time PCR analysis

Gene	Primer name	Sequence (5' to 3')	Application
Trout IL-34	IL-34 F1	CCAAAGAAAGTGAGGCTTCAGGGA	3'-RACE
	IL-34 F2	ACGGGGGTTTTACTCTGGGTGTTG	3'-RACE
	IL-34F	AGGCAGAAGACGTAACATGAAACACA	Real-time PCR
	IL-34R	CCACCCTCGCCCTCAGCTT	Real-time PCR
Fugu IL-34	fIL-34F1	CGGCGCTACATGAAACACTA	3'-RACE
	fIL-34F2	CCCCATCAACTACACCATCA	3'-RACE
	fIL-34R1	CTTCAACACCCCCTGGTAGA	5'-RACE
	fIL-34R2	GAAGCAGAACCTCCACCTGT	5'-RACE
Trout EF-1α	EF-1αF	CAAGGATATCCGTCGTGGCA	Real-time PCR
	EF-1αR	ACAGCGAAACGACCAAGAGG	Real-time PCR
Trout MCSF1	MCSF1F	AAGACTGAGCCAAACCATCCTAGGAC	Real-time PCR
	MCSF1R	GGATAAGGGCTTGGAGTCTCTTCTTCTC	Real-time PCR
Trout MCSF2	MCSF2F	CCTCCCTACAGCACTCTCTCTGACTAC	Real-time PCR
	MCSF2R	GGTCAGTACTGTAGGACATCTTGTGTGT	Real-time PCR

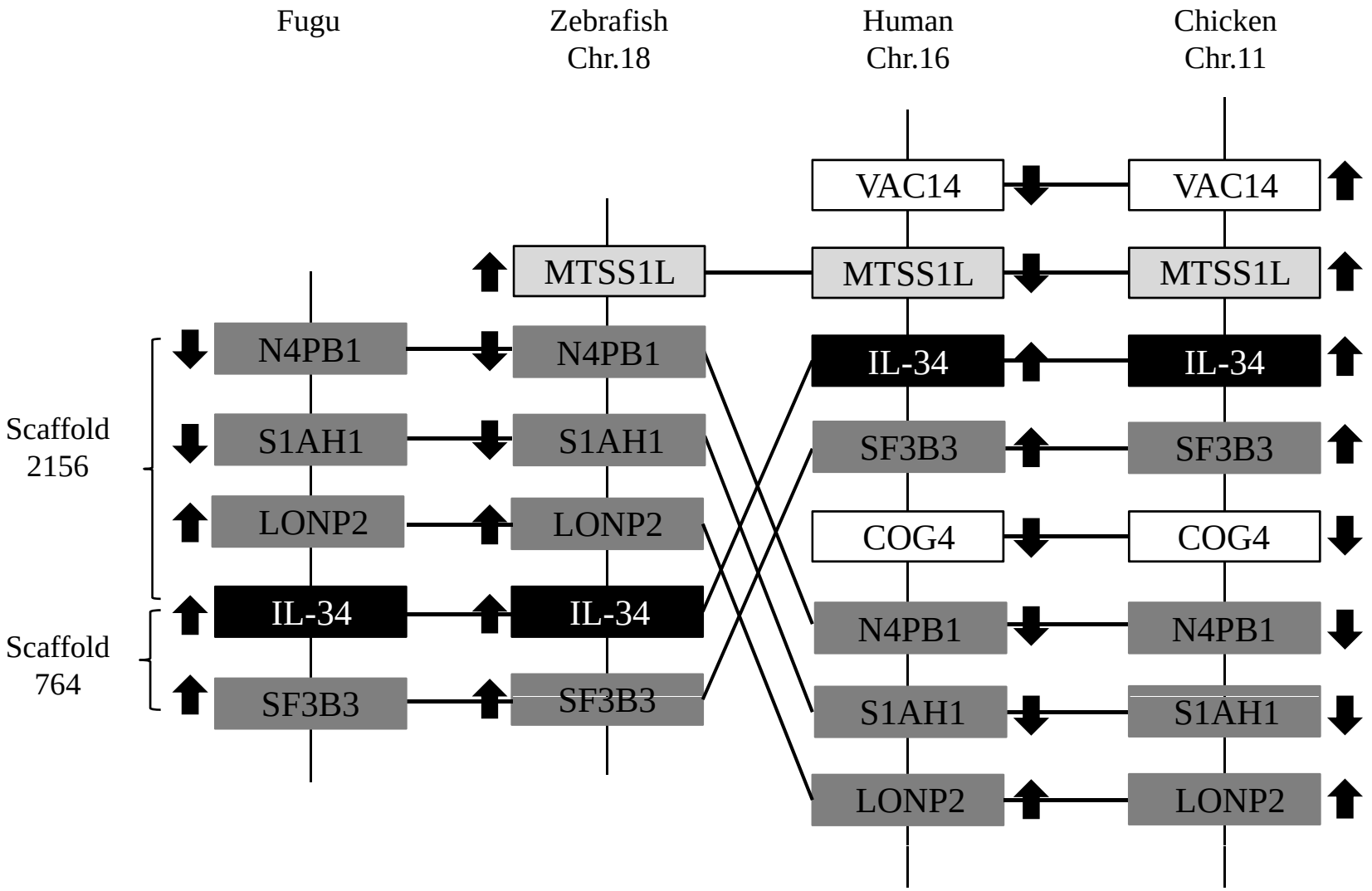
Table 2. Summary of teleost IL-34. The signal peptide predicted using SignalP 4.0 program is shaded and potential-glycosylation sites are underlined. The number of amino acids (aa), and the theoretical isoelectric point (pI) and molecular mass (MM) of the full-length translation are presented.

Molecule	Amino acid sequence	aa/pI/MM	Evidence
Trout IL-34	MVRSTAWLLGALLGLICVLPLVLMTPRTAQCTSLKTLENKLIGRRRN MKHNLPI**NYT**IRVHYEEVFKLS**NIS**KLRARVEDLEDGDLQDVWLLVN REVLKRILRVLPVRHPSYKYTTDLEDLFRKVQQVFPPQTDEREPPER IEEIYKRVKEIDSKGWRFVTPKSLLDNCYRTMHCLFKDCFSSEDREQ DYCGLPHWRKGRKRLQ	204/ 24.31/ 9.42	Cloned in this study. Supplementary Fig. 1.
Salmon IL-34A	MVRSTAWLLGALLGLIYVLPSVLMTPRTAQCTSLKTLENKLIGRRRN MKHNLPI**NYT**IRVHYEEVFKLS**NIT**RLRARVEDLEDGDLQDVWLLVN QEVLKRILRVLPVRHPSYKYTTDLEDLFRKVQQVFPTQSDEREPPER IEEIYKRVKEIDSKGWTFVTPKSLLDNCYRTMHCLFKDCFPSEDREQ DYCGLPHWRKGRKRLL	204/ 24.29/ 9.30	Predicted from a contig of 28 ESTs that matched WGSs: AGKD01002735 andAGKD01091083. Supplementary Fig. 3.
Salmon IL-34B	MVRPTSLLLGGLFGLMWVIPVLMTPTTLAQCTSLKTLETKLTDRRRN LKHNFPI**NYT**IRVHYEELFKLS**NIS**RLRVRVDDLEEGDLQDVWLLVN QEVLKRILRVLPVRHPSYKYTSDLEDLFRKIQQVFPPQSDEREPPER IEEIYNRVKEPNSKGWRFVTPKSLLDNCYRTMHCLFKNCFPSEDGEQ DYCSSLHWRKGRKRQLQAT	207/ 24.61/ 9.22	Predicted from WGS:AGKD01156379. Supplementary Fig. 4.
Fugu IL-34	MVQLVTSVYLLGGLWGLFLLGPTAQTPSSMCTPLKTINDSLSHRRRY MKHYFPI**NYT**IRVHAYEVFRLS**NIS**RMRPQVEVLLLQQLWFQVYQGV LKKIIRVLSERHPSRSYTAELERRFQDAEGVFVQSHPVEVFQQELPE AIQETWDHLTEDPERVPESRWRYASPKALLDNLCYTMHCLFRECFPS TELQQDYCSFSQWRKGRKKPDQQEGDVVLDDCGEESDS	226/ 26.61/ 6.18	Cloned in this study. Supplementary Fig. 2.
Catfish IL-34	MVRFETWLLLVLLGLMWALPVWMSFPSPSPISKNSPLCTSLVTLKDQ L**NSS**LRRRYLKHNFPI**NYT**IHVRYEEVFRLK**NIS**RMK**NDS**EIEKHLQ DVWVDVTVTVIQSILNVLPERHPTRHKYLANLESLLKAFQTIWVKTD ESYYTENIFNIVKHLGMEKYEARKSVRPKSLLDNCYRTMHCLFKDCF LR**NSS**QDDYCDTQHWRKV**NGT**QG	211/ 25.08/ 9.27	Predicted from a contig of 6 ESTs. Supplementary Fig. 5.
Zebrafish IL-34	MVQSECWLLRGLLGFICLLPVCSSAAPDLCGPLKTVQDSL**NAT**LRRR YMKMHFPI**NYT**VQVRYEEVFRLR**NIS**RLV**NTS**NEEEPVLPRDLQDLW LYVSQQGIKKVLRVLPERHPTRRKYLSDLENLFKKFETVFKEGNHED QENVRERPESLQTIWDHLTEQDYKGWKSVTPKSILDNCYRTMLCLFK ECFTKEDDNYDYCEVYNRRKERKTT	213/ 25.51/ 8.36	Predicted, B3DLJ8, and matched 6 ESTs
Tilapia IL-34	MVQLSTAVCLLGGLFLIAPVLMAPTHSSMCTPLRTI**NDS**LSHRRQYM KHNFPIEYTIKVHNREIFRLS**NIS**RMRLRTEGLNELVLQRLWFQVYQ GVLKKILWVLPTRHPSRPYTAELERRFKDAQAVFMQSHPAQVFQEDL PEKIHDIWDSLTEKPENMPESSWRFATPKSLLDNLCRTMYCLFSECF SNADVQEDYCEVSHWRKGRKKDMQPES	215/ 25.36/ 8.74	Predicted, I3JZ08

Table 3. Comparison of identities (top right) and similarities (bottom left) of teleost IL-34 molecules with selected IL-34 sequences from birds and mammals. The accession numbers of the sequences used are as in Fig. 3.

	1	2	3	4	5	6	7	8	9	10	11	12
1. Trout		95.1	82.2	44.0	47.2	46.5	49.3	27.1	33	31.1	28.9	27.8
2. Salmon-A	97.1		82.2	44.8	46.8	46.0	49.3	29.8	33.2	31.2	29.3	28.5
3. Salmon-B	89.4	88.9		47.2	49.8	46.8	48.4	27.2	31.0	30.0	26.2	24.5
4. Fugu	60.6	61.9	65.5		63.6	37.4	41.6	27.7	31.5	27.8	29.7	26.0
5. Tilapia	65.1	64.2	67.0	76.5		41.0	43.9	31.2	32.4	25.6	22.2	22.6
6. Catfish	60.2	59.7	63.5	52.2	57.2		47.3	29.8	31.8	31.4	27.6	26.4
7. Zebrafish	66.2	64.8	65.7	58.8	60.9	65.7		28.8	33.8	26.6	28.5	25.8
8. Chicken	47.1	47.1	46.9	41.6	45.1	47.4	45.5		76.7	30.6	33.3	31.5
9. Finch	49.5	49.0	49.8	41.2	45.6	46.9	48.8	85.0		32.9	36.1	33.9
10. Human	45.0	44.6	47.1	40.5	41.7	48.8	42.1	42.6	45.0		71.9	68.2
11. Cow	44.0	44.0	43.2	42.3	37.6	46.6	43.6	45.7	48.3	81.4		67.8
12. Mouse	43.0	44.3	44.3	41.3	38.7	47.7	43.8	44.7	46.0	78.5	78.7	

Fig. 1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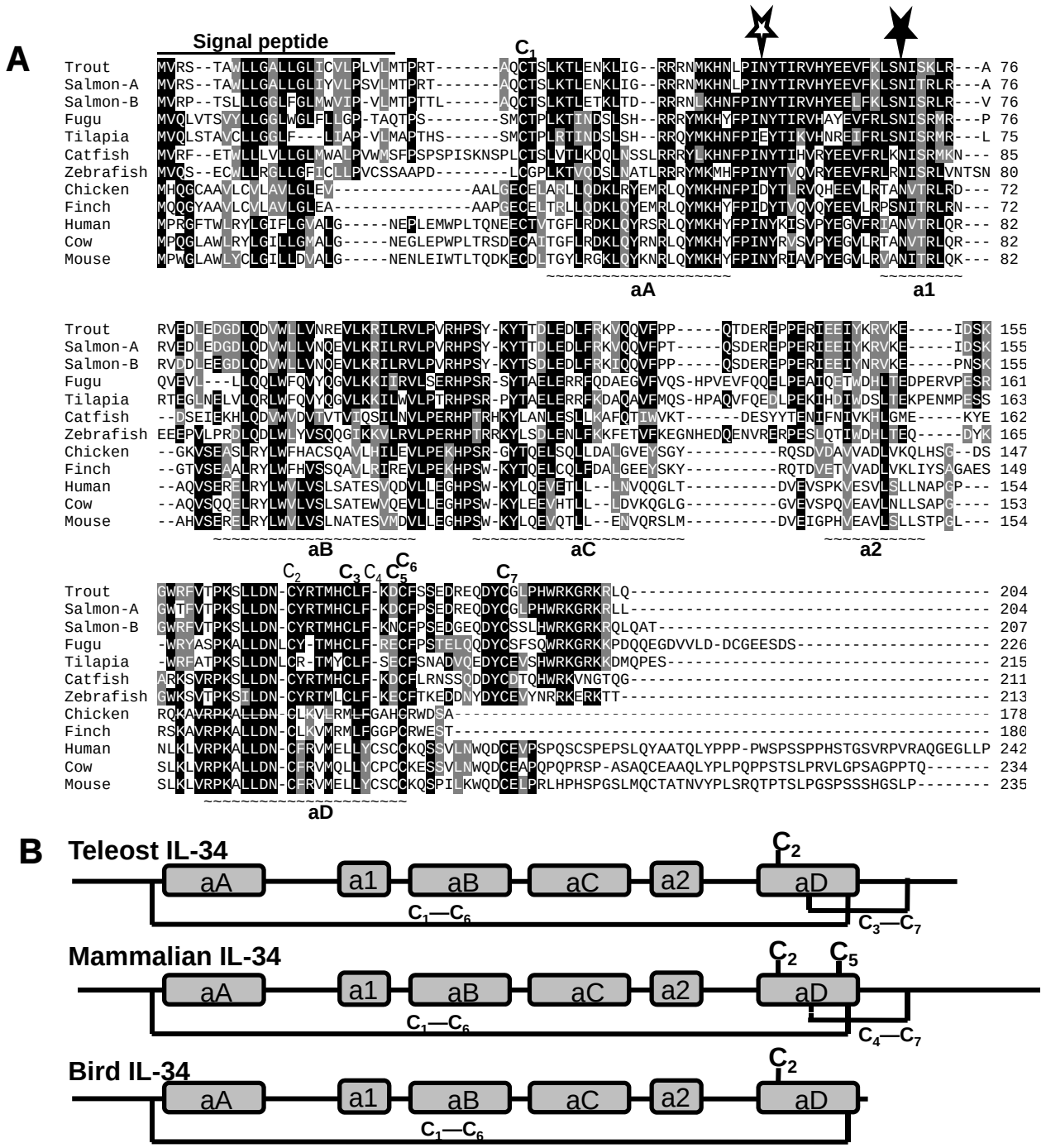
Zebrafish
Chr.18
Human
Chr.16
Chicken
Chr.11
Scaffold
2156
Scaffold
764
VAC14
MTSS1L
N4PB1
S1AH1
LONP2
IL-34
SF3B3
COG4

Fig. 2


A
Signal peptide
C1
Trout
Salmon-A
Salmon-B
Fugu
Tilapia
Catfish
Zebrafish
Chicken
Finch
Human
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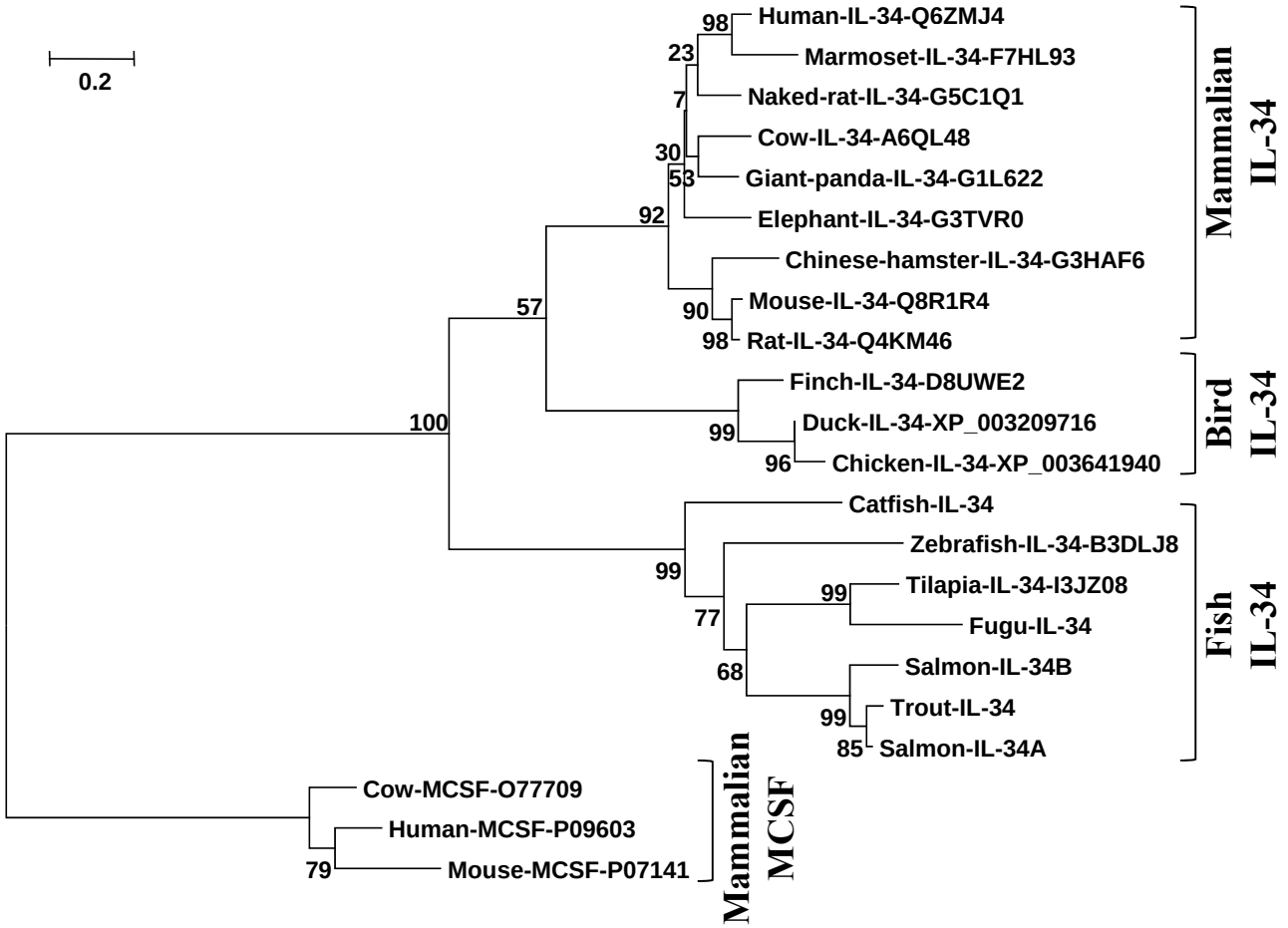
Mouse
aA
a1
aB
aC
a2
C2
C3
C4
C5
C6
C7
aD
B
Teleost IL-34
Mammalian IL-34
Bird IL-34
C1—C6
C3—C7
C4—C7

Fig. 3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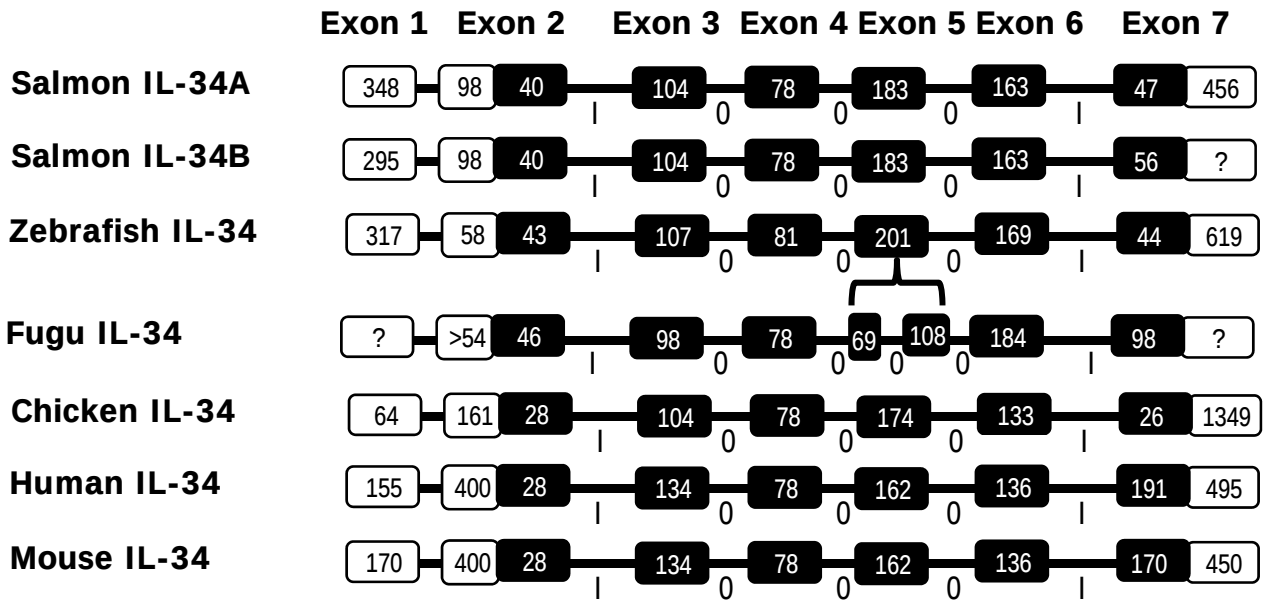
Cow-IL-34-A6QL48
Giant-panda-IL-34-G1L622
Elephant-IL-34-G3TVR0
Chinese-hamster-IL-34-G3HAF6
Mouse-IL-34-Q8R1R4
Rat-IL-34-Q4KM46
Finch-IL-34-D8UWE2
Duck-IL-34-XP_003209716
Chicken-IL-34-XP_003641940
Catfish-IL-34
Zebrafish-IL-34-B3DLJ8
Tilapia-IL-34-I3JZ08
Fugu-IL-34
Salmon-IL-34B
Trout-IL-34
Salmon-IL-34A
Cow-MCSF-O77709
Human-MCSF-P09603
Mouse-MCSF-P07141
98
23
7
30
53
92
57
90
98
100
99
96
99
77
99
68
99
85
79
Mammalian IL-34
Bird IL-34
Fish IL-34
Mammalian MCSF

Fig. 4


Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
Exon 7
Salmon IL-34A
348 98 40 104 78 183 163 47 456
Salmon IL-34B
295 98 40 104 78 183 163 56 ?
Zebrafish IL-34
317 58 43 107 81 201 169 44 619
Fugu IL-34
? >54 46 98 78 69 108 184 98 ?
Chicken IL-34
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Human IL-34
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Mouse IL-34
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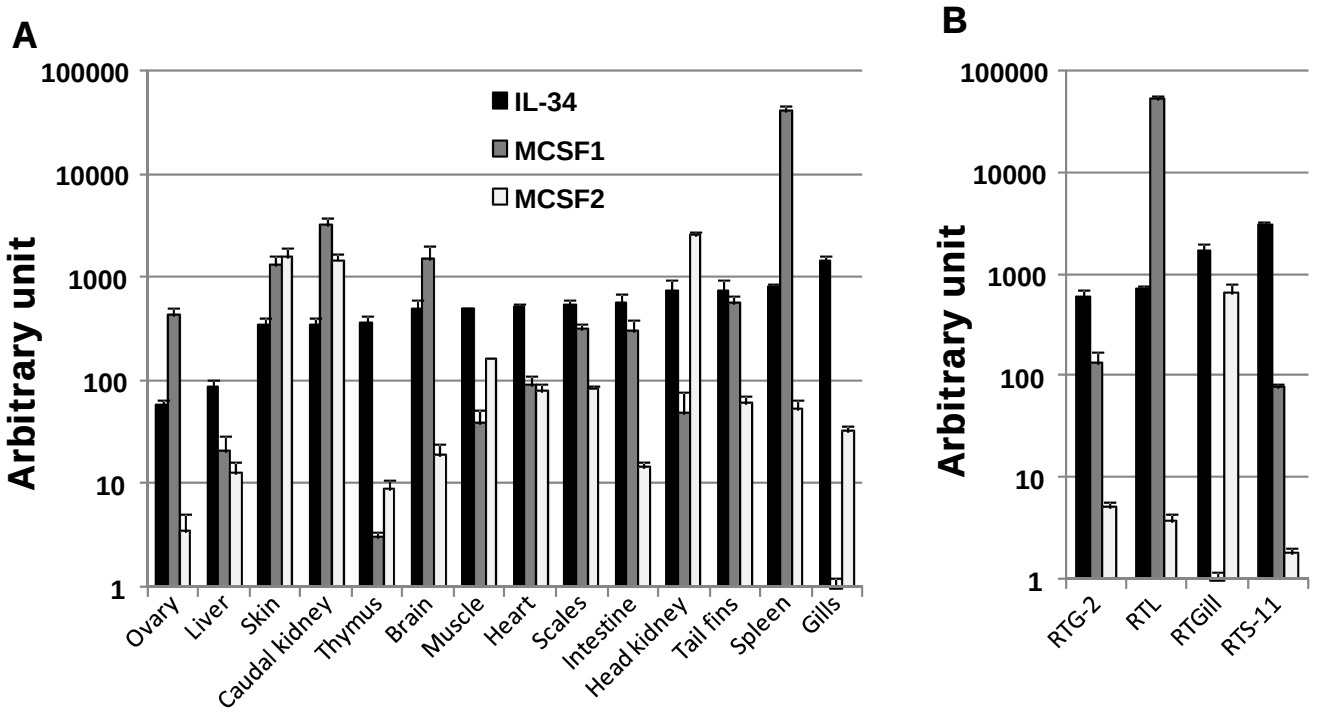
Fig. 5



C

	Ovary	Liver	Skin	CK	Thymus	Brain	Muscle	Heart	Scales	Intestine	HK	Tail fins	Spleen	Gills	RTG-2	RTL	RTGill	RTS-11
IL-34/MCSF-1	0.13	4.24	0.26	0.11	121.30	0.33	12.80	5.71	1.70	1.86	15.04	1.29	0.02	1415.99	4.51	0.01	1694.00	39.86
p-value	.001	.011	.001	.001	.000	.408	.000	.003	.005	.011	.005	.657	.000	.000	.003	.000	.000	.000
IL-34/MCSF-2	16.70	6.94	0.22	0.24	41.42	26.14	3.13	6.42	6.48	39.72	0.29	12.27	15.27	43.63	117.14	189.07	2.56	1671.05
p-value	.000	.000	.001	.000	.000	.000	.000	.000	.000	.000	.003	.002	.000	.000	.000	.000	.000	.000
MCSF-1/MCSF2	127.18	1.64	0.84	2.19	0.34	79.97	0.24	1.12	3.80	21.31	0.02	9.48	757.62	0.03	25.95	14447.34	0.00	41.92
p-value	.000	.540	.774	.044	.025	.002	.001	.834	.000	.001	.003	.000	.000	.000	.000	.000	.009	.000

Fig. 6

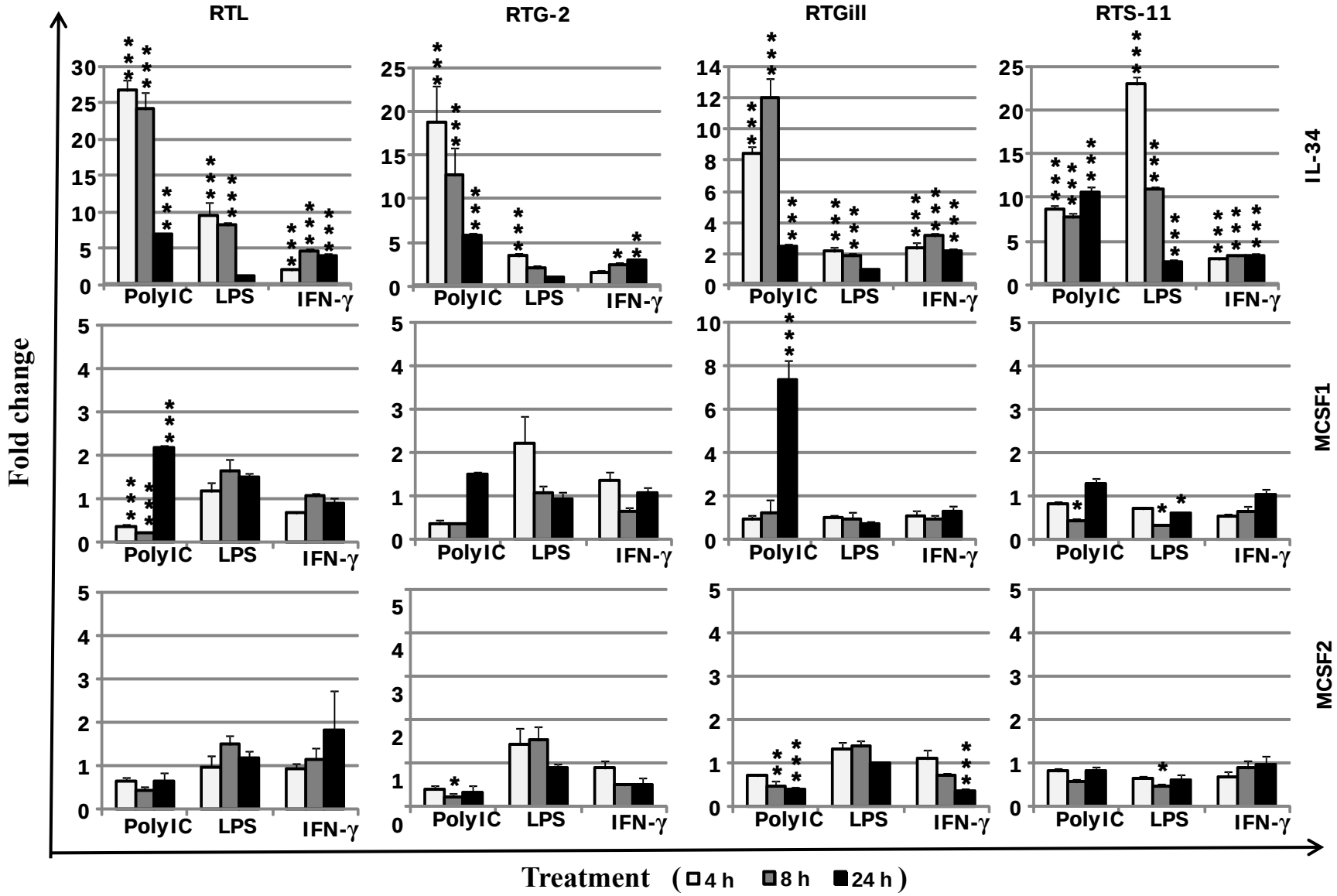

RTL
RTG-2
RTGill
RTS-11
IL-34
MCSF1
MCSF2
Fold change
PolyIC
LPS
IFN-γ
Treatment (4 h 8 h 24 h)

Fig. 7

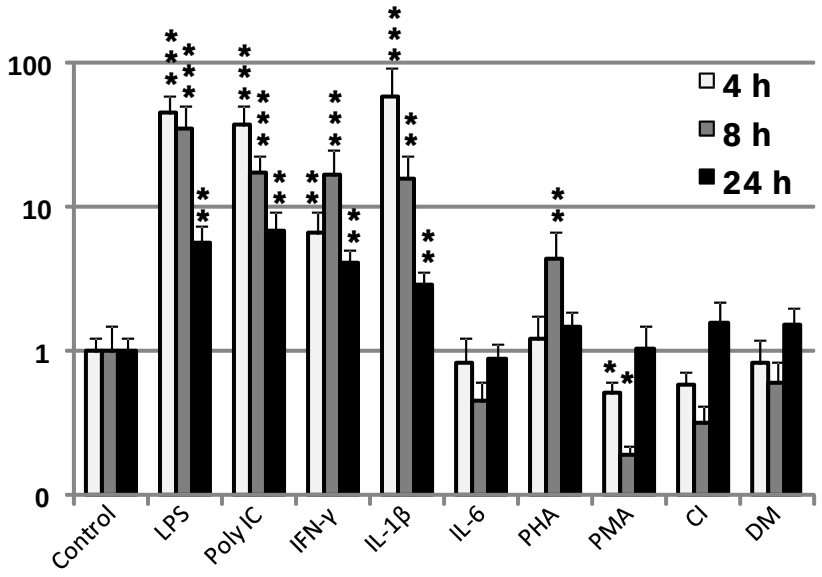
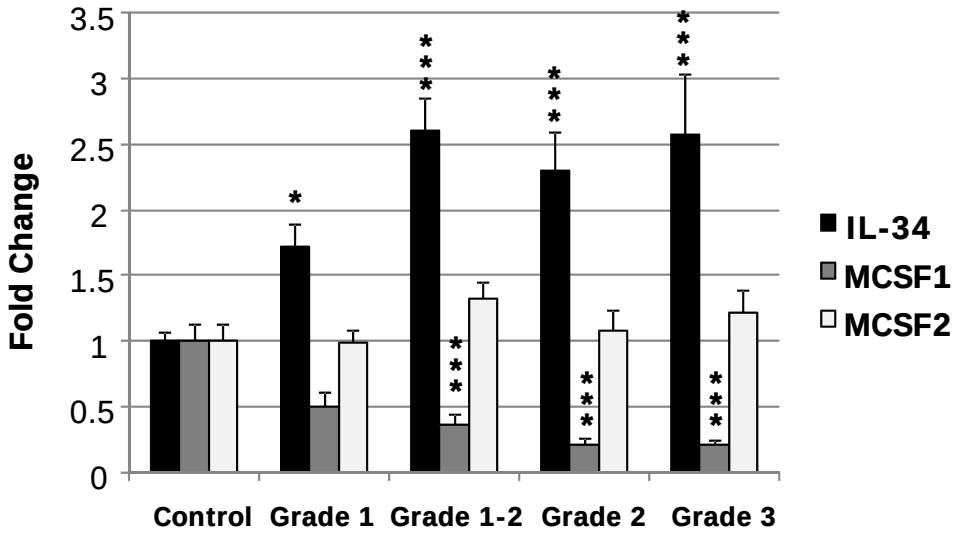

100
10
1
0
4 h
8 h
24 h
Control
LPS
PolyIC
IFN-γ
IL-1β
IL-6
PHA
PMA
CI
DM

Fig. 8


Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
Control
Grade 1
Grade 1-2
Grade 2
Grade 3
IL-34
MCSF1
MCSF2