Characterisation and expression analysis of the Atlantic halibut (*Hippoglossus hippoglossus* L.) cytokines: IL-1β, IL-6, IL-11, IL-12β and IFNγ

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Abstract Genes encoding the five Atlantic halibut (*Hippoglossus hippoglossus* L.) cytokines; interleukin (IL)-1β, IL-6, IL-11b, IL-12βc, and interferon (IFN) γ, were cloned and characterised at a molecular level. The genomic organisation of the halibut cytokine genes was similar to that seen in mammals and/or other fish species. Several mRNA instability motifs were found within the 3′-untranslated region (UTR) of all cytokine cDNA sequences. The putative cytokine protein sequences showed a low sequence identity with the corresponding homologues in mammals, avian and other fish species. Nevertheless, important structural features were presumably conserved such as the presence, or absence in the case of IL-1β, of a signal peptide, secondary structure and family signature motifs. The relative expression pattern of the cytokine genes was analyzed in several halibut organs, revealing a constitutive expression in both lymphoid and non-lymphoid organs. Interestingly, the gills showed a relatively high expression of IL-1β, IL-12βc and IFNγ. The real time RT-PCR data also showed that the mRNA level of IL-1β, IL-6, IL-12βc and IFNγ was high in the thymus, while IL-11b was relatively highly expressed in the posterior kidney and posterior gut. Moreover, the halibut brain showed a relatively high level of IL-6 transcripts. Anterior kidney leucocytes in vitro stimulated with imiquimod showed a significant increase in mRNA level of the five halibut cytokine genes. The sequence and characterisation data presented here will be useful for further investigation of both innate and adaptive immune responses in halibut, and be helpful in the design of vaccines for the control of various infectious diseases.

Introduction

For a numbers of years, farming of the marine flatfish Atlantic halibut (*Hippoglossus hippoglossus* L.) has been of commercial interest; however, it is yet to progress beyond the establishing phase. A poorly developed larva at hatching and a relatively long live feed stage has given problems, making the halibut larvae vulnerable to bacterial and viral diseases associated with high mortalities [1]. Better knowledge about the halibut immune system is thereby important, as it will facilitate the establishment of adequate prophylactic counter measures such as vaccines and the use of probiotics. Cytokines are important modulators of the vertebrate immune system, and could be helpful in the study of both innate and adaptive immune responses. Since little is known about the halibut cytokine network, effort was made to identify expressed sequence tags (EST) representing cytokines in a database created on the basis of Atlantic halibut cDNA libraries [2]. This resulted in the identification of ESTs resembling the

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cytokine genes interleukin (IL)-1β, IL-6, IL-11, IL-12β and interferon (IFN)γ.

In mammals, IL-1β is a pleiotropic cytokine regulating numerous immune and inflammatory responses. IL-1β is one of the earliest to be expressed amongst the pro-inflammatory cytokines, promoting a cascade of reactions leading to inflammation, many of which depend on the regulation and expression of other cytokines and chemokines. IL-6 is one of the inflammatory cytokines induced by IL-1, exhibiting both pro-inflammatory and anti-inflammatory properties. Like IL-1, IL-6 is also a pleiotropic cytokine involved in the regulation of processes such as immunoglobulin (Ig) synthesis, T-cell differentiation, acute phase reaction, hematopoiesis and neuro-endocrine processes. Another member of the mammalian IL-6 family is IL-11, which shares the same receptor subunit, glycoprotein 130 (gp130), as IL-6. A pleiotropic property with an involvement in inflammatory processes and hematopoiesis has also been designated to this cytokine. IL-12, another pro-inflammatory cytokine, is critical in the defence against parasites, viruses and intracellular bacteria. It stimulates the production of IFNγ, mostly in natural killer (NK) cells and T-cells, and is important in the regulation of the cell-mediated immune response by enhancing the proliferation and cytolytic activities of NK- and T-cells. In mammals, IFNγ is the only member of the type II class of IFNs, regulating the transcription of several hundred genes, having various immunoregulatory functions in both innate and adaptive immunity. In addition to being a so-called key T_H1 cytokine, it is also important in macrophage activation and enhancement of phagocytosis, in regulation of cell proliferation and apoptosis, and the promotion of peptide antigen presentation.

In teleost, IL-1β is probably the most widely studied of the known cytokines, and has been characterised in a number of fish species [3–13]. Like its mammalian counterpart, teleost IL-1β have been found to be regulated in response to various stimuli [4, 6–18], and the biological activity of recombinant IL-1β (rIL-1β) has been studied in several fish species indicating that fish IL-1β is involved in the regulation of immune relevant genes, lymphocyte activation, migration of leucocytes, phagocytosis and bactericidal activities [7, 15, 19–24]. The first IL-6 sequence identified in teleosts was reported in fugu (*Takifugu rubripes*) [25], and further characterised in fish species such as Japanese flounder (*Paralichthys olivaceus*), rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) [26–28]. Increased expression of IL-6 has been shown in response to different stimuli [25–28], and indirect evidence has been found for a tumor necrosis factor (TNF) α and IL-1β-induced expression of IL-6 in flounder [26]. The teleostean IL-11 orthologue has been found to consist of a duplicated fish IL-11 gene, named IL-11a and IL-11b [29],

with expression patterns indicating that both divergent forms of teleostean IL-11 play roles in antibacterial and antiviral defence mechanisms of fish [29–31]. Similar to IL-11, distinct forms of IL-12β and IFNγ have been identified in fish. IL-12β has been well characterised in sea bass (*Dicentrarchus labrax* L.), fugu and common carp (*Cyprinus carpio*) [32–34], where three distinct IL-12β genes (type a, b and c) have been sequenced in common carp and later retrieved from the genomes of zebrafish (*Danio rerio*) and fugu [32, 33]. However, the induction of IL-12β expression in response to in vitro stimuli has been found to vary between the different forms of IL-12β and between different fish species. Two distinct, but closely linked, teleostean IFNγ genes have been found [35–42], one having a conserved C-terminal nuclear localization sequence (NLS) (IFNγ or IFNγ2), and one atypical type without this important feature (IFNγrel or IFNγ1). IFNγ expression in fish suggests an active role in both innate and adaptive immune responses, as up-regulation in response to parasite, bacterial and viral pathogens and mitogens has been detected at the transcriptional level, in addition to induction by recombinant TNFα and in mixed leukocyte reactions [35, 37, 39–41, 43].

This study reports the cloning and characterisation of these five cytokines in halibut, together with the basal gene expression pattern in several organs of Atlantic halibut. Moreover, the modulation of cytokine transcript level was analysed in anterior kidney leucocytes in vitro stimulated with imiquimod, a imidazoquinoline compound shown to be a potent inducer of several cytokines and IFNs in mammals [44]. Extended knowledge about the gene organisation of these cytokines in another taxonomic order of fish (*Pleuronectiformes*), and new valuable insight in the constitutive expression level of fish cytokines by the use of real time RT-PCR is here given. This information will be helpful in the further study of Atlantic halibut immune responses, and will be valuable in the fight against disease, such as nodavirus, hampering the halibut production.

Materials and methods

Fish stocks and sample collection

Fish were acclimatised upon arrival at IMR (Bergen), Norway, reared in 9°C sea water (salinity of 34.5‰) and fed commercial feed twice a day. If injected, fish were anesthetised with benzocain (The Norwegian medicine depot) at a concentration of 60 mg/l seawater, while for tissue sampling an overdose of benzocain was employed.

Ten individuals, approximately 1 year old weighing between 70 and 150 g, were obtained from Austevoll Aquaculture Research station, IMR, Norway. Samples

were collected from thymus, spleen, anterior and posterior kidney, pectoral fins, gills, brain, eye, anterior and posterior gut, red and white muscle, skin, heart and liver from four fish for total RNA isolation. The organ samples were snap-frozen in liquid nitrogen immediately after dissection and stored at $-80°C$ until use. From the six remaining fish, anterior kidney leucocytes were isolated for in vitro study of cytokine expression as described in "Leucocyte isolation for the in vitro study" section.

To identify the 5′ end of IFNγ (RACE), anterior kidney leucocytes were isolated from four fish, approximately 6 months old and weighing approximately 30 g, obtained from Aga Marine, Bømlo, Norway. Two fish were injected intra peritoneal (i.p) with 200 μl of L-15 medium (Sigma), while the other two were injected i.p with 200 μl $1 \times 10^{8.5}$ $TCID_{50}$ nervous necrosis virus (NNV) and leucocytes were isolated 10 weeks post injection as described previously [2]. The isolated leucocytes were further stimulated with 10 μg/ml ConA (Calbiochem) in combination with 5 ng/ml PMA (Calbiochem) (ConA-PMA). Cells were harvested at 4 h post stimulation, pelleted and frozen at $-80°C$ until use.

Leucocyte isolation for the in vitro study

Anterior kidney leucocytes for the in vitro study were isolated using discontinuous percoll gradients as previously described for Atlantic cod (Gadus morhua) [45], with some modifications as follows. The centrifugation steps were performed at 400 g for 40 min (percoll gradient) and 10 min (wash step). Only one wash step was performed by re-suspending the cell pellet in a complete salt minimal essential medium (CSMEM), prepared as described by Patel et al. [2]. The leucocytes were counted using Glasstic slides with grids (Hycor Biomedical), and cell viability evaluated using 0.02% Trypan Blue Stain (Gibco).

The leucocytes were plated out in 24-well culture plates (Falcon), having approximately 3×10^6 cells/ml in each well. Control cells and cells stimulated with 3 μg/ml imiquimod (Calbiochem) were incubated at 15°C for 6, 12, and 24 h post stimulation. To harvest the leucocytes, the CSMEM medium was removed and centrifuged for 10 min at 400 g, 15°C to pellet the non-adherent cells. TRI reagent (Sigma), 1 ml, was thereby added to the adherent cells within each well, transferred to the pellet of non-adherent cells after repetitive mixing with the pipette, and frozen at $-80°C$ until it was used for total RNA isolation.

Isolation of total RNA and cDNA synthesis

Total RNA from organ samples was isolated using TRI reagent (Sigma) according to the Trizol reagent protocol described by Invitrogen, with a few modifications as described previously [46]. Total RNA from leucocytes was purified by a combined Trizol (Invitrogen) and RNAeasy (Qiagen) method, as previously described [47]. However, for the in vitro study the total RNA was isolated using the RNeasy Micro kit. Briefly, the aqueous phase from the chloroform phase separation (Trizol method) was added 1 volume of 70% ethanol, transferred to an RNeasy Micro spin column, and total RNA was further purified according to the RNeasy® Micro Handbook (Qiagen).

The concentration and the purity of the total RNA were assessed with a NanoDrop Spectrophotometer (NanoDrop Technologies), and the quality of random samples was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was reverse transcribed using a Reverse Transcription Core Kit (Eurogentec) and random nonamers as primers in 30 μl reactions with 500 ng (tissue) or 300 ng (anterior kidney leucocytes) total RNA. The cDNA was stored at $-20°C$ until use.

DNA sequencing and bioinformatic analysis

EST representing IL-1β (GenBank accession no.: GE628686), IL-6 (GenBank accession no.: GE628244), IL-11b (GenBank accession no.: GE628883), IL-12βc (GenBank accession no.: GE628376), and IFNγ (GenBank accession no.: GE628285) were identified by blast search in a database created on the basis of Atlantic halibut cDNA libraries [2]. To sequence the 5′ end and 3′ end of the IL genes, the SMART™ RACE cDNA amplification kit from Clontech was used as described previously [48]. SMART™ RACE cDNA to identify the 5′ end of IFNγ was performed using a pool of total RNA (348 ng) from the ConA-PMA stimulated leucocytes. Amplification and sequencing of cDNA was performed so as to confirm the open reading frame (ORF) of the genes, and genomic sequences were amplified to confirm exon–exon boundaries, as previously described [46].

The ORFs were blasted using ExPASy BLAST form (http://ca.expasy.org/tools/blast/), and aligned with ClustalW (www.ebi.ac.uk/Tools/clustalw/index.html). Location of domains was predicted using InterProScan (http://www.ebi.ac.uk/InterProScan), and physico-chemical parameters were calculated using ProtParam (http://au.expasy.org/tools/protparam.html). Post translational modifications were predicted using the NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Secondary structure prediction was done using the PSIpred protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/).

Real time RT-PCR assay and data analysis

Primers and probes for real time RT-PCR were designed and the PCR efficiency analysed as previously described

Table 1 Primers and probes used for real time RT-PCR analysis

Gene	Forward	Reverse	Probe	E%
IL1β	TCAGAGATGAAAGCCTGCTCAA	GATCTGGTGGAGACGAGCTTCT	TGGAAGAGCACATTGTGTT-MGB	97
IL6	GGAATTCATCCTCAAACTCTTGCT	TAAGCGCCTCACCGATCTG	TTACACTGATGATCAAGCC-MGB	99
IL11b	TCCTGAAGTTTGTTTCCCACAGT	CTGGTGCAAGTCTCCAAAACAC	TCTCTGGCAGCCCATGA-MGB	94
IL12βc	TGAGGATGAAGAGGAATTAGCTCAAT	CACTTTCTTTCAAGGTACAGCTGAAG	TTGTTTGGCAAAGTCT-MGB	101
IFNγ	TGAGGAGGCATCGCTACCA	TCGCCCAATGCCTTGCT	AAGTCCAGATGGATGACC-MGB	99

The primers and probes are listed 5′ → 3′ direction with the corresponding PCR efficiency (E%). All probes, besides IL-1β that was marked with VIC, are marked with 6-carboxyfluorescein (6FAM)

MGB minor groove binder

[47]. The primer and probe sequences with corresponding PCR efficiencies are listed in Table 1. The PCR reaction mix contained 1× TaqMan Fast PCR Master Mix (Applied Biosystems), 900 nM of each primer, 200 nM TaqMan probe and 1 µl cDNA in a final volume of 12.5 µl. The PCR cycling was carried out as follows: 95°C for 20 s, 40 cycles of 95°C for 1 s followed by 60°C for 20 s. Samples were run in duplicate on the 7900 HT Fast Real-Time PCR System (Applied Biosystems), and the mean Ct value for each sample was used for analysis if the deviation was smaller than 5%. The real time RT-PCR data was normalised using elongation factor 1 alpha (EF1A1) as internal reference gene [47]. Efficiency (E) of each assay was taken into consideration and the relative expression was transformed using the following formula $E^{-\Delta\Delta Ct}$ [49, 50], calibrated against liver or muscle expression. The mRNA level in anterior kidney leucocytes in vitro stimulated with imiquimod was related to the non-stimulated control cells ($E^{-\Delta Ct}$), and significant regulation was analysed with the non-parametric Mann–Whitney U test.

Results and Discussion

Characterisation and expression analysis of IL-1β

Amplification of genomic DNA using primers designed based on the halibut IL-1β cDNA sequence, revealed a sequence of 2,522 base pairs (bp) consisting of five exons separated by four introns (GenBank accession no.: FJ769830) (Fig. 1). Comparing the halibut IL-1β gene organisation with other IL-1β genes confirms what is seen in other vertebrates, with a conserved exon–intron pattern at the 3′ end within the three last exons, not seen at the 5′ end. The human and carp IL-1β genes have seven exons separated by six introns [51, 52], and in fish species such as rainbow trout and Atlantic cod the IL-1β gene have been found to be separated into six exons [9, 11, 53]. However,

orange-spotted grouper (*Epinephelus coioides*), sea bass, sea bream, and tilapia (*Oreochromis niloticus*) have a similar organisation to that of halibut, with five exons [3, 6–8]. Generally, the first exon remains un-translated, and differences are seen between the next one, two, or three exons. Buonocore et al. [3] have argued that sea bass IL-1β, having a similar genomic organisation as halibut IL-1β, has merged the second and third exon (corresponding to trout exon numbering). The discovery of this particular organisation in halibut, which belongs to the *Pleuronectiformes*, indicates that this is likely to be seen in other species belonging to the *Percomorpha*.

The halibut IL-1β cDNA sequence of 1,255 nucleotides (nt) (GenBank accession no.: FJ769829), consisted of a 70 nt long 5′-untranslated region (UTR), and a 444 nt long 3′-UTR. The 3′-UTR contained four ATTTA motifs, typically found in cytokine mRNA to mediate RNA instability [54]. An ORF of 741 nt encoding 246 amino acids (aa) was deduced within the halibut IL-1β cDNA sequence, showing resemblance to other sequences with sequence identities between 79 and 31% (Table 2). The highest conservation of halibut IL-1β is seen within the regions of the 12 predicted β-sheets (Online resource 1A), indicating the presence of a β-trefoil structure as seen in the mammalian members of the IL-1 family. This β-trefoil folding is very important for receptor binding, forming a cluster of the amino acids contacting the IL-1RI receptor [55]. These residues are poorly conserved in the deduced sequences encoding halibut and other teleost IL-1β [3–13], indicating the involvement of other amino acids in the interaction between fish IL-1β and IL-1Rs. Alike most teleost IL-1β sequences [3–13], the IL-1 family signature motif [FS]-x_2-[FYLIV]-[LI]-[SCA]-T-x_7-[LIVM] is reasonably well conserved in halibut IL-1β.

The halibut IL-1β lacks a signal peptide, indicating a non-classical secretion pathway for the halibut IL-1β such as for other mammalian and non-mammalian counterparts. In mammals, IL-1β is stored in the cytoplasm as a 31 kDa

Fig. 1 Schematic representation showing the genomic organisation of the halibut cytokine genes. *Black boxes* represent 5′- and 3′-UTR, *white boxes* represents exons while the introns are indicated with a *line*. In IL-11b, the *stippled line* represents the position and length of a putative intron
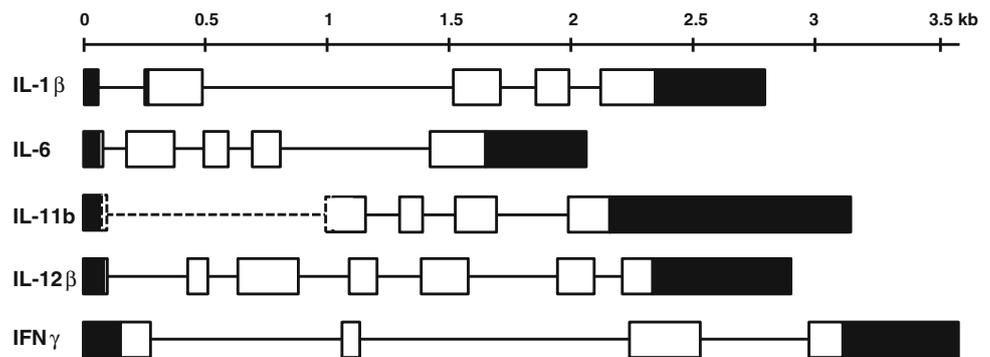

Table 2 Feature summary of the halibut cytokines

	IL-1β	IL-6	IL-11b	IL-12βc	IFNγ
Exon/intron	5/4	5/4	?/?	7/6	4/3
ATTTA	4	5	4	6	9
ORF (amino acids)	246	226	201	301	206
Signal peptide	–	1–24	1–26	1–21	1–23
Mature peptide					
Molecular mass (kDa)	?	23.0	19.8	31.7	20.9
Potential O-Glyc	–	2	–	2	1
Potential N-Glyc	2	1	4	3	1
Signature motif	$LxSAx_2Px_2YISTx_7V$	$Cx_9Cx_6GLx_2Yx_3F$	Leucine and proline rich	WSxWT	$IQxKAx_2ELx_2L$
Secondary structure	12 β-sheets	4 α-helical bundle	4 α-helical bundle	Ig-fold, fibronectin-type III	6 α-helixes
Amino acid similarities					
Flounder (%)	79	86	73 (b)	–	65
Fugu (%)	–	50	32 (a), 53 (b)	26 (a)	42
Rainbow trout (%)	54	30	28 (a)	50 (c)	33
Common carp (%)	34	–	28 (a)	34 (c), 25 (b), 24 (a)	27
Zebrafish (%)	32	–	29 (a), 27 (b)	35 (b), 29 (a)	23
Chicken (%)	32	23	–	26	17
Mouse (%)	32	19	21	27	12
Human (%)	31	21	21	26	13

The different divergent forms of IL-11 and IL-12β are given in *brackets* after the percent identities. The lack of published sequences within a fish species are indicated with a *dash*

inactive precursor. Generally, the caspase IL-1β converting enzyme (ICE) cleaves Aspartate-X bonds (X designates a small hydrophobic residue), converting the cytokine into its 17 kDa active form [56]. It is believed to be released upon stimuli by the shedding of IL-1β containing micro-vesicles [57, 58]. As seen in halibut (Online resource 1A), other characterised fish, amphibian and bird IL-1β sequences lack a clear ICE [3–13, 59]. From amino acid alignments, possible mature peptide has been predicted in trout and sea bass [12, 24], and found to have biological activity in vivo and in vitro [19, 24]. Furthermore, a 24 kDa mature peptide has been detected upon stimulation of a trout macrophage cell line [60], and a 22 kDa mature form of sea bream IL-1β was found to be released from SAF-1 fibroblasts in a similar manner as seen in mammals [61]. The presence of an aspartate residue within the halibut IL-1β sequence (D^{86}) aligning with the proposed cutting site in sea bass (Online resource 1A), though followed by the hydrophilic amino acid glutamine, supports this. An arginine (R^{80}) followed by a serine (S^{81}) is also found within the halibut IL-1β sequence and in similar species such as Japanese flounder (GenBank accession no.: AB070835) and turbot (*Psetta maxima*, GenBank accession no.: AJ295836), similar to the cleavage site of the calcium-dependent protease calpain in murine IL-1β [62]. However, the poor sequence conservation within this region makes it difficult to conclude, and suggest that even different fish species within different phylogenetic groups may have evolved distinct cutting strategies.

Characterisation and expression analysis of IL-6

A genomic IL-6 sequence of 1,829 bp was amplified as overlapping fragments, consisting of five exons separated by four introns (GenBank accession no.: GU985454) (Fig. 1). The genomic organisation of halibut IL-6 was similar to what is seen in mammals and other fish species [25, 26, 28, 63, 64]. The size of the exons was also found to be comparable. However, differences are seen, e.g., between the second and last exons of halibut and fugu [25], and between the last exon only when compared to the closely related flounder [26].

The halibut IL-6 cDNA sequence (Genbank accession no.: GU985455) was found to be 1,147 nt long, having a 5′-UTR of at least 289 nt, and a 187 nt long 3′-UTR with five mRNA instability motifs. An ORF of 681 nt encoding a protein of 226 aa was deduced from the cDNA sequence, having a sequence identity ranging from 86 to 30% with other teleosts, and typically between 29 and 19% with higher vertebrates (Table 2). The halibut IL-6 sequence was predicted to display a 4 α-helical folding classifying it as a class-I helical cytokine (Online resource 1B). Thus, it is likely to have a core structure consisting of a closed bundle of four helices in a left-handed twist with two crossover connections, as seen in mammals [65]. The fifth α-helix seen in mammalian IL-6 in the final long loop between helix C and D, was also predicted within the halibut IL-6. However, as in the other fish IL-6's characterised to date [25–28], halibut IL-6 lack the two first (corresponding to human C^{44} and C^{50}) out of four cysteines believed to stabilize the 4 α-helical fold. These N-terminal cysteines were found to be less important for the biological activity of IL-6 in human than the last two cysteines (corresponding to human C^{73} and C^{83}) [66]. C^{73} and C^{83} are part of the IL-6/G-CSF/MGF family consensus pattern (C-x$_9$-C-x$_6$-G-L-x$_2$-[FY]-x$_3$-L) that is reasonably well conserved between fish and mammals [25–28], including halibut (Online resource 1B). However, in halibut IL-6 the aliphatic leucine has been substituted by the aromatic phenylalanine, as seen in flounder IL-6 [26]. Three interaction regions are believed to be important for receptor binding and signal transduction within mammalian IL-6 [67]. The ligand specific receptor (IL-6R) is thought to bind through a site I, while two binding sites have been suggested for the transmembrane signal transducer gp130 (site II and III), also involved in the receptor complex for other type I cytokines including IL-11. These regions are, however, poorly conserved between human and fish (Online resource 1B), though the identification of halibut IL-6R and gp130 may give further insight into the understanding of IL-6 signalling mechanisms in halibut.

Characterisation and expression analysis of IL-11b

Primers were designed based on a halibut IL-11 cDNA sequence showing resemblance to the fish IL-11 type b, and

genomic IL-11b DNA was amplified revealing a partial sequence of 1,033 bp consisting of four exons separated by three introns (GenBank accession no.: GU985456) (Fig. 1). Presumably, the first intron was not successfully sequenced; however, it is likely to be situated seven bp from the start codon (Fig. 1), as seen in zebrafish, the green spotted pufferfish (*Tetraodon nigroviridis*), fugu and mammals [29]. If so, this gives halibut IL-11b a genomic organisation with exon sizes similar to what is seen in the other fish IL-11b.

The halibut IL-11b cDNA sequence (Genbank accession no.: GU985457) was found to be 1,663 nt long, having a 5′-UTR of at least 76 nt, and a 3′-UTR of 981 nt with four mRNA instability motifs. An ORF of 606 nt encoding a protein of 201 aa was found, having a sequence identity ranging from 73 to 26% with other teleostean IL-11b, and around 21% with mouse and human (Table 2). When comparing the halibut IL-11b with known IL-11a sequences, identities of around 32–28% is found.

Like IL-6, the halibut IL-11 sequence was predicted to display the 4 α-helical folding, classifying it as a class-I helical cytokine (Online resource 1C). Similar to mammalian IL-11, the deduced protein was rich in proline (5.7%) and leucine (16.6%), also seen in other teleost IL-11's [29–31]. Due to the lack of cysteines, the 4 α-helix bundle structure in mammalian IL-11 is believed to be stabilized merely by hydrophobic interactions. However, as in other teleost IL-11's, the halibut IL-11b possess cysteines that may form stabilizing disulphide bridges, although, they are not fully conserved. For instance, a cysteine in helix D seen in all IL-11a's and IL-11b's sequenced to date was substituted by a glycine in halibut (Online resource 1C).

A high number of positively charged residues (9 arginine and 11 lysine) were found within the halibut IL-11b, in addition to the polar amino acids histidine, threonine and serine, positioned in the α-helices in a way that they were likely to be exposed to the surface of the protein. The theoretical isoelectric point was indicated to be 9.67 for the mature halibut IL-11b, giving it a basic nature as for the mammalian IL-11's and fish IL-11a's. The fugu IL-11's have a pI of 8.56 and 9.17 for IL-11b and IL-11a, respectively. As for IL-6, a hexameric receptor complex is also proposed to be found for mammalian IL-11, existing of two IL-11R's, two IL-11's, and two gp130 molecules [68]. Interestingly, an arginine (corresponding to human R^{169}) and a tryptophan (corresponding to human W^{166}), shown to be important for IL-11R binding in mammals, are preserved in some or all teleostean IL-11's, respectively, including halibut IL-11b (Online resource 1C).

Characterisation and expression analysis of IL-12βc

Primers were designed based on a halibut IL-12β cDNA sequence showing resemblance to the type c IL-12β's of

fish, and was further used to amplify halibut IL-12βc genomic DNA. This revealed a sequence of 2,620 bp, separated into seven exons divided by six introns (GenBank accession no.: FJ769832) (Fig. 1). The genomic organisation of halibut IL-12βc was somewhat different from that seen in the type a IL-12β's of zebrafish, sea bass and fugu that have an eight exon seven intron organisation, as seen in human [33, 34]. However, chicken has the same seven exon six intron organisation as halibut IL-12βc [69]. Moreover, variations in exon sizes and the number of coding exons are seen, as the fugu IL-12β is encoded by eight exons, sea bass, zebrafish, halibut and mouse are encoded for by seven exons, while human and chicken are only encoded for by six exons.

The halibut IL-12βc cDNA sequence (GenBank accession no.: FJ769831) consisted of 1,548 nt, with a 5′-UTR of at least 83 nt and a 642 nt 3′-UTR having six mRNA instability motifs. The ORF of 906 nt encoded a 301 aa long putative protein showing the highest resemblance to the type c IL-12β's of common carp [32], rainbow trout (GenBank accession no.: AJ548829) and Atlantic salmon (*Salmo salar*, GenBank accession no.: BT049114), with sequence identities between 50 and 34% (Table 2). As for other IL-12β's, an Ig-like domain (D1) and two fibronectin-type III domains (D2 and D3) were predicted within the halibut IL-12βc (Online resource 1D), with the highly conserved WSxWS signature motif seen as WSxWT in D3 of halibut IL-12βc. The cysteine pair within D1 and the first pair of cysteines within D2, found to form intra-chain disulphide bonds in human IL-12β, were conserved in all available IL-12β sequences. The second disulphide cysteine pair of D2 was only conserved in the type a and b of fish IL-12β's available at GenBank, as only the first of the two cysteines was conserved in IL-12βc's, and those of D3 were not conserved in any of the available teleostean IL-12β's. Though, other cysteines are found that could form alternative disulphide bonds within the fish IL-12β's. A cysteine corresponding to human C^{177} found to form an inter-chain disulphide bond with IL-12α, is not found in the IL-12βc's, but seen in type a and b. Though, mutational analysis has indicated that this disulphide bond is not crucial for dimerization, but rather ensuring a stable association [70]. Upon IL-12 heterodimerization, charged residues E^{181} and D^{290} of human IL-12β are found to interact with opposite charged and hydrophilic residues of IL-12α within a hydrophobic binding pocket formed by hydrophobic residues of IL-12β [70]. Residues found to be essential for the formation of this pocket are either conserved or substituted by other hydrophobic residues within the teleost IL-12β's, including halibut IL-12βc. Additionally, E^{181} and D^{290} are conserved within all the IL-12β sequences available at GenBank, indicating a formation of a similar binding pocket in teleostean IL-12β.

Characterisation and expression analysis of IFNγ

Amplification of genomic DNA using primers designed based on the halibut IFNγ cDNA sequence, revealed a sequence of 3,051 bp consisting of four exons separated by three introns (GenBank accession no.: GU985450) (Fig. 1). The halibut IFNγ gene organisation is fairly similar to what is seen in other fish, avian and mammalian IFNγ genes [35, 36, 40, 71], however, with a third exon that is somewhat longer in the halibut sequence. This is also, to a lesser extent, seen in other teleostean IFNγ sequences. Suggested from the amino acid alignment (Online resource 1E), additional nt are seemingly found at the beginning of the third exon, not seen in other teleosts, and in the middle, as seen in other teleosts. Generally, it seems like the lower the level of phylogeny, the fewer nt have apparently been added.

The halibut cDNA sequence found to be similar to IFNγ (GenBank accession no.: GU985451) was 1,237 nt long, having a 5′-UTR of at least 149 nt, and a 3′-UTR of 467 nt with nine mRNA instability motifs. An ORF of 621 nt encoding a putative protein of 206 aa was deduced, having a sequence identity ranging from 65 to 23% with other teleostean IFNγ, and around 12–13% with mouse and human IFNγ (Table 2). The putative halibut IFNγ was predicted to have a 4 α-helical bundle structure, apparently missing one of the six α-helixes of mammalian IFNγ (Online resource 1E). Though, additional helixes were predicted within the elongated CD loop of halibut within exon 3, and in the C-terminal end as seen in cod IFNγ [40]. The signature motif, [IV]-Q-x-[KQ]-A-x_2-E-[LF]-x_2-[IV], was conserved within the helix corresponding to human helix F, also seen among the other known IFNγ sequences [35–41]. Moreover, the NLS, important for nuclear translocation and the biological activity of IFNγ, is seemingly conserved in the C-terminal region of the teleost IFNγ2 sequences [35–41], including halibut IFNγ (Online resource 1E). In rainbow trout, a deletion of this motif resulted in loss of activity [37], indicating a preserved function of this cationic NLS motif. In mammals, the biologically active IFNγ is a homodimer of two mature IFNγ molecules, interacting with a receptor tetramer of two IFNγR1 and two IFNγR2 molecules. The IFNγ homodimer is non-covalently associated having a globular structure where four of the helixes of one monomer are interdigitated with two of the helices of the other monomer [72]. As the helical structure is conserved within the teleost IFNγ molecules, including halibut IFNγ (Online resource 1E), a similar dimer association is possible. The human IFNγ dimer is contacting the IFNγR1 receptor unit only with residues within helix A and the AB loop as well as with residues within helix F. These residues in halibut IFNγ are poorly conserved, except for the residues within helix F

corresponding to human K^{108}, E^{112}, and A^{118} that are a part of the IFNγ signature motif (Online resource 1E). These residues are thus likely to be important for receptor association in fish as well.

Basal expression pattern of the cytokine genes in halibut organs

In mammals, IL-1, IL-6, IL-11, IL-12 and IFNγ is expressed by a variety of cell types, either constitutively or produced upon stimulation. For instance, IL-1, IL-6, IL-11 and IL-12 are found to be expressed in the thymus, either by the developing T-lymphocytes or by the stromal cells. Neutrophils, monocytes and macrophages are found to express IL-1, IL-6, and IL-12. Macrophages are additionally, together with other professional antigen presenting cells (APCs) such as dendritic cells (DCs) and B-cells, found to express IFNγ in addition to IL-12. NK- and NKT-cells are found to constitutively express IFNγ, while CD4$^+$ T$_H$1 cells, CD8$^+$ T cytotoxic (T$_C$) -cells and APCs produce IFNγ only upon induction. The basal expression of halibut IL-1β, IL-6, IL-11b, IL-12βc and IFNγ do in many ways support a similar expression pattern as in mammals, however, furher immunohistochemical and functional studies should be conducted to illustrate wich cell types are expressing the halibut cytokines. However, by means of real time RT-PCR, the importance of the different cytokines within the halibut tissues can be estimated.

The mRNA level of IL-1β was evident in both immune organs and organs believed to not be directly involved in immune responses (Fig. 2). A constitutive expression of IL-1β has also been observed in various organs of orange-spotted grouper [7], carp [18, 51], sea bream [8], sea bass [10], and channel catfish (*Ictalurus punctatus*) [13], likely to reflected the pleiotropic nature of this cytokine. Expression of IL-11 in mammals has also been detected in a wide range of normal adult tissue, evident for the expression of halibut IL-11b as well with variable levels of IL-11b mRNAs detected in several of the analysed tissues. Seemingly, the basal expression in tissues was quite low for Japanese flounder IL-11b [31]; however, not in the expression of carp and rainbow trout IL-11as that had a more homogenous expression pattern amongst the tissues tested [29, 30]. IL-6 on the other hand has been reported to be scarcely produced under normal circumstances and rather rapidly and transiently up-regulated during pathogenic invasion or in relation to stress in mammals. In accordance with this, the mRNA level was generally quite low in most organs analysed in halibut, as seen for other teleost IL-6 forms analysed [25–28], yet relatively high levels detected in the halibut thymus and brain. Generally, a relatively high mRNA level of halibut IL-12βc was detected in immune related organs only, while lower levels

was detected in both immune organs and organs not directly involved in immune responses. A constitutive expression of fugu IL-12βa and common carp type a, b, and c was also detected in several organs [32, 34], supporting this. A low constitutive expression of IFNγ was detected in all the halibut organs analysed in this study, as has been detected in lymphoid and non-lymphoid organs of other teleost species [38–41, 43].

Relatively high levels of halibut IL-1β, IL-6, IL-12βc and IFNγ where detected in the thymus, while a moderate transcript level of IL-11 was seen. As seen in higher vertebrates the teleostean thymus is believed to be the site for T-cell maturation [73], however, the knowledge about the action of cytokines in the regulation of T-cell development in fish is poor. In mammals, IL-1 and IL-6 is produced by reticulo-epithelial (RE) cells within the stromal cellular microenvironment [74], cells producing numerous cytokines important in different stages of hematopoietic cell activation and differentiation. Thymic IL-12 expression in mammals has been connected to alteration of thymocyte migration, differentiation, and cell death in combination with IL-2 and IL-18 and the production of IFNγ [75]. IFNγ produced by T-lymphocytes during T-cell development has been shown to induce Ag presentation and apoptosis [76–79]. Thereby, the relatively high expression of IL-1β, IL-6, IL-12βc and IFNγ in the halibut thymus demands for further functional and biological studies as to analyse the importance of these cytokines in T-cell maturation of fish.

The halibut gill did also show relatively high levels of IL-1β, IL-12βc and IFNγ transcripts. The expression of other immune related genes have been observed in gills of halibut [46, 48, 80–82], even the recombination activating gene (RAG1) responsible for T-cell receptor rearrangement [83]. Moreover, a novel interbranchial lymphoid tissue was suggested to be situated in the gills of rainbow trout and Atlantic salmon [84, 85], indicating that the cytokine expression in the halibut gills may be connected to immune cells situated in the gills for surveillance. Naturally, the fish would benefit from an active defence system within the gills being the respiratory organ of fish, not only by the expression of key inflammatory cytokines (IL-1β), but also cytokines important in cell-mediated immunity (IL-12β and IFNγ). In rainbow trout, common carp, cod and goldfish, the mRNA level of IFNγ in gills was also shown to be relatively high compared to other organs tested [39–41, 43]. Further investigation of this would thus be of great interest, also in regard to the fin expression.

The expression of IL-6 was also found to be relatively high in the halibut brain (Fig. 2), supported by the expression of IL-6 in the trout brain as well [27]. In mammals, IL-6 participate in the development and function of the central nervous system [86]. Both IL-1β and IL-6 are shown to be involved in the hypothalamus–pituitary–adrenal (HPA) axis and the stress response in mammals
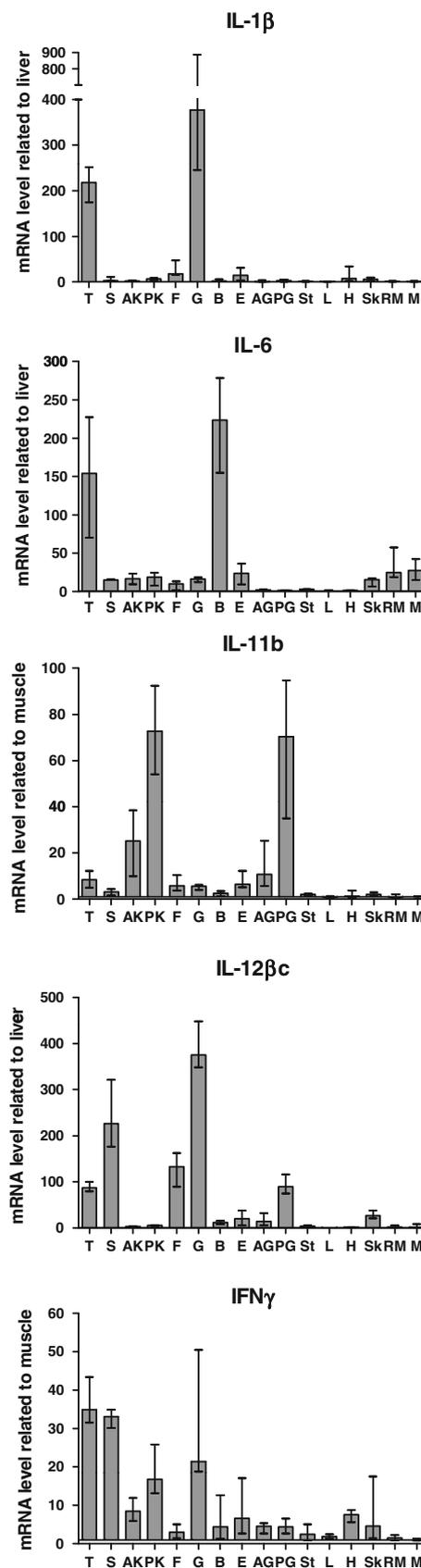
Fig. 2 Relative level of the halibut cytokine mRNAs analysed in ▶ different halibut organs by the means of real time RT-PCR. Elongation factor 1α (EF1A1) served as internal reference gene while the organ showing the lowest expression (liver for IL-1β, IL-6 and IL-12βc, and white muscle for IL-11b and IFNγ) was used as calibrator. Data represents median values of $n = 4$ fish (±interquartile range). *Abbreviations*: *T* thymus, *S* spleen, *AK* anterior kidney, *PK* posterior kidney, *F* pectoral fins, *G* gills, *B* brain, *E* eye, *AG* anterior gut, *PG* posterior gut, *St* stomach, *RM* red muscle, *M* white muscle, *Sk* skin, *H* heart and *L* liver

[87]. Interestingly, IL-6 has been connected to long-term stress responses in mammals [87], and thus further elucidation of the IL-1β and IL-6 function in the halibut brain would be very interesting. Moreover, a lower transcript level of IL-12βc in the halibut eye and brain was detected, also seen in the brain of fugu and common carp [32, 34].

Generally, the halibut kidney showed a relatively low expression of the analysed cytokines, however, relatively high levels of halibut IL-11b was detected, especially in the posterior part. In mammals, IL-11 is involved in the regulation of the hematopoiesis as it stimulates the proliferation and differentiation of primitive stem cells, as well as multipotent and committed progenitor cells [88]. As the teleostean kidney is believed to be the site for hematopoietic differentiation, including erythropoiesis, granulopoiesis, and lymphopoiesis, a similar importance of fish IL-11 in hematopoiesis could be expected. This is supported by findings in carp where IL-11a showed a higher expression in posterior kidney compared to anterior kidney [29]. Though, only lower levels of IL-11b transcripts were found in the kidney of Japanese flounder [31], and rainbow trout IL-11a was highly expressed in anterior kidney; however, posterior kidney was not included in this study [30]. It should also be considered that the kidney of teleosts is involved in immune responses functioning as a secondary lymphoid tissue [89].

Expression of the cytokine genes in in vitro stimulated anterior kidney leucocytes

In mammals, imidazoquinoline compounds as imiquimod has been shown to be a potent inducer of several cytokines and IFNs, including IL-1β, IL-6, IL-12 and IFNγ [44]. In mammals, imiquimod induced cytokine expression is likely to be activated by its interaction with toll-like receptor (TLR) 7 [90]. TLR7 recognises single stranded viral RNA, and is exclusively embedded in endosomal membranes of some immune cells [91–93]. Consequently, when stimulated with imiquimod or derivatives TNFα, IL-1β and IL-6 expression has been found to be increased in mammalian monocytes [94], elevated levels of TNFα, IL-6 and IL-12 have been reported in macrophages [90], while plasmacytoid DCs are found to be the predominant cell type producing IFNα [95]. Moreover, a high production of IFNγ has
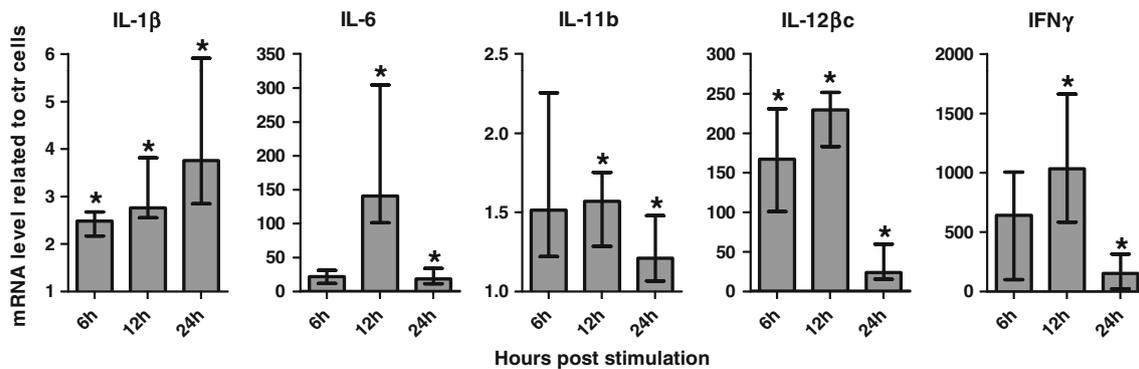
Fig. 3 Relative level of the halibut cytokine mRNAs in anterior kidney leucocytes in vitro stimulated with imiquimod for 6, 12 and 24 h. The mRNA level was measured by the means of real time RT-PCR, and the mRNA level in the stimulated cells was related to the mRNA level in control cells. Data is represented as median values ($n = 6$) $\pm$ interquartile range. A significant increase in mRNA level was analysed with the non-parametric Mann–Whitney U test and is indicated with an *asterisk* ($P < 0.05$)

been observed in imiquimod stimulated human peripheral blood mononuclear cells (PBMCs) and mouse spleen cell cultures, likely to be expressed by T-cells as a response to elevated levels of IFNα and IL-12 produced by monocytes/macrophages [96].

In accordance with this, halibut anterior kidney leucocytes in vitro stimulated with imiquimod showed a significant increase in mRNA level of IL-1β, IL-6, IL-11b, IL-12βc and IFNγ (Fig. 3). Both IL-1β and IL-11b showed a rather moderate increase in mRNA level at the three time points tested. Still, a relatively high increase in the transcript level of IL-6 was detected 12 h post imiquimod addition, as well as high levels of IL-12βc and IFNγ mRNAs at 6 and 12 h post stimulation. Both pro-inflammatory cytokines like TNFα, IL-1β, IL-8, IL-10 and IL-12, as well as IFNs and IFN stimulated genes (ISG) like IFNα, IFNβ, IFNγ, ISG15 and Mx, have been reported to be up-regulated in response to imiquimod in fish [97–99], supporting the increased expression of the halibut cytokine genes. As seen in the present study, higher levels of IFN transcripts (IFNα1) when compared to the inflammatory cytokines tested (TNFα, IL-1β and IL-8) were observed in imiquimod stimulated trout anterior kidney leucocytes [98]. Together, these results indicate that a similar mechanism of imiquimod related induction of cytokine expression as seen in mammals could be expected in teleost fish as well.

Conclusion

Similar to other piscine cytokine genes, the halibut IL-1β, IL-6, IL-11b, IL-12βc and IFNγ are poorly conserved. Despite the low amino acid similarity, the halibut cytokine sequences show a somewhat preserved gene organisation and structural conservation, giving strong evidence that the identified cytokine molecules are orthologue equivalents to their mammalian counterparts. The basal expression pattern of the halibut cytokines suggests a possible function of IL-1β, IL-6, IL-12βc and IFNγ within the thymic microenvironment. On the other hand, IL-11b seems to be more important within the kidney. The detection of relatively high levels of IL-1β, IL-12βc and IFNγ within the halibut gills underlines the importance of the halibut gills in immune surveillance and would be an interesting story to pursue. Moreover, the involvement of the halibut cytokines in neuro-endocrine processes, especially IL-1β and IL-6, should be of interest for further investigation. Supported by results in other fish species [97–99], the enhanced mRNA level of the halibut cytokine genes, especially IL-6, IL-12 βc and IFNγ, in anterior kidney leucocytes in vitro stimulated with imiquimod, indicates a possible application for imidazoquinoline compounds as antiviral therapeutics and vaccine adjuvants of fish.

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