Identification, molecular characterization and functional analysis of interleukin (IL)-2 and IL-2like (IL-2L) cytokines in sea bass (*Dicentrarchus labrax* L.)

Francesco Buonocore[1*], Marco Gerdol[2], Alberto Pallavicini[2], Valentina Stocchi[1], Elisa Randelli[1], Maria Cristina Belardinelli[1], Andrea Miccoli[1], Paolo Roberto Saraceni[1], Christopher J. Secombes[3], Giuseppe Scapigliati[1], Tiehui Wang[3]

[1]Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Largo dell'Università snc, 05100 Viterbo (VT), Italy.
[2]Department of Life Sciences, University of Trieste, Via Giorgieri 5, 34127 Trieste (TS), Italy.
[3]Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK.

*Corresponding author at: Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Largo dell'Università snc, 05100 Viterbo (VT), Italy, E-mail address: fbuono@unitus.it

ABSTRACT

In mammals, interleukin (IL)-2, initially known as a T-cell grow factor, is an immunomodulatory cytokine involved in the proliferation of T cells upon antigen activation. In bony fish, some IL-2 orthologs have been identified, but, recently, an additional IL-2like (IL-2L) gene has been found. In this paper, we report the presence of these two divergent IL-2 isoforms in sea bass (*Dicentrarchus labrax* L.). Genomic analyses revealed that they originated from a gene duplication event, as happened in most percomorphs. These two IL-2 paralogs show differences in the amino acid sequence and in the exon 4 size, and these features could be an indication that they bind preferentially to different specific IL-2 receptors. Sea bass IL-2 paralogs are highly expressed in gut and spleen, which are tissues and organs involved in fish T cell immune functions, and the two cytokines could be up-regulated by both PHA stimulation and vaccination with a bacterial vaccine, with IL-2L being more inducible. To investigate the functional activities of sea bass IL-2 and IL-2L we produced the corresponding recombinant molecules in *E. coli* and used them to *in vitro* stimulate HK and spleen leukocytes. IL-2L is able to up-regulate the expression of markers related to different T cell subsets (Th1, Th2 and Th17) and to Treg cells in HK, whereas it has little effect in spleen. IL-2 is not active on these markers in HK, but shows an effect on Th1 markers in spleen. Finally, the stimulation with recombinant IL-2 and IL-2L is also able to induce *in vitro* proliferation of HK- and spleen-derived leukocytes. In conclusion, we have demonstrated that sea bass possess two IL-2 paralogs that likely have an important role in regulating T cell development in this species and that show distinct bioactivities.

Keywords: interleukin-2; sea bass; expression; bioactivity; T cell development

1. INTRODUCTION

Interleukin (IL)-2 is one of the most extensively studied cytokines. Its importance as a growth-promoting factor for T lymphocytes has been known since 1976 [1] and few years later it became one of the first human cytokines to be studied at a molecular level [2]. Mammalian IL-2 is active on many different cell types, mainly T lymphocytes, and one of its fundamental functions is to promote proliferation of both $CD4^+$ and $CD8^+$ T cells [3]. Moreover, it has been demonstrated that IL-2, together with IL-15, acts as a growth factor for natural killer (NK) cells [4] and that it can increase antibody secretion from activated B cells [5]. Studies carried out on IL-2 knock out mice have shown that IL-2 is able to downregulate immune responses, mainly to prevent autoimmunity [6], but recent data have revealed that this cytokine also has a pivotal role in immune tolerance [7]. IL-2 production is tightly regulated, and, in mammals, it is mainly expressed by activated $CD4^+$ T cells [8]. Hence, IL-2 expression can be induced *in vitro* in T cells by T cell mitogens, such as phytohemoagglutinin (PHA) [9].

Mammalian IL-2 is a monomeric glycoprotein with a molecular weight of about 15 kDa. Its crystal structure has been known since 1992 [10] and consists of a four α-helical bundle (from A to D) folded in an "up-up-down-down" configuration typical of short-chain type I cytokines. A single disulphide bond connects the second helix to the inter-helical region between the third and the fourth helix. The high affinity IL-2 receptor (IL-2R) is formed by three subunits, IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (CD132, known as γC). The crystal structure of the complex formed by IL-2 and its high-affinity trimeric receptor has provided insights into the way the receptor chains assemble. This process seems to be guided by IL-2 itself, since the first interaction with IL-2Rα leads to a small conformational change in the cytokine, which promotes its association with IL-2Rβ. Finally, γC is added to the complex through a strong interaction with IL-2Rβ, aided by a weaker interaction with IL-2 [11]. The signal transduction of the quaternary complex inside the cell is mediated by the tyrosine kinases Jak1 and Jak3 [12]. Successively, the MAPK and PI3K kinase pathways are activated, which lead to a modulation of gene expression through the translocation of the Stat5 transcription factor to the nucleus [7, 13].

In fish, IL-2 was first identified in Fugu (*Takifugu rubripes*) by exploiting the conservation of synteny between the human and Fugu genome [14]. Subsequently, IL-2 has been cloned in rainbow trout (*Oncorhynchus mykiss*) [15-16], where its bioactivity has been investigated [15, 17-18], and in the large yellow croaker (*Larimichthys crocea*), where a

recombinant molecule has been produced in yeast [19]. In both fish species the recombinant IL-2 protein was able to up-regulate the expression of genes involved in Th1 (IL-2, IFNγ) and Th2 (IL-4/13A and IL-4/13B), but not Th17 (different isoforms of IL-17) type immune responses. In contrast to mammals, a second gene copy of IL-2 has been discovered in several fish genomes. In some instances, this is due to a duplication of the IL-2 locus, which appears to have occurred on multiple occasions during fish evolution. For example, based on chromosomal organization and phylogenetic evidence, the two IL-2 genes found in salmonids and carps are thought to be the direct result of an additional (4R) whole-genome duplication (WGD) event that occurred independently in these two lineages. On the other hand, the IL-2-like (IL-2L) gene found only in some percomorphs (e.g. *Takifugu rubripes*, *Tetraodon nigroviridis* and *Gasterosteus aculeatus*), is in close proximity to the IL-2 gene, suggesting its origin was a relatively recent lineage-specific local tandem gene duplication in the IL-2 locus [20-22]. A recent paper has investigated the biological activity of the two rainbow trout IL-2 paralogs, shedding some light on the functional divergence of these duplicates [23].

In this report, we cloned and characterized structurally two IL-2 genes (IL-2 and IL-2L) in sea bass (*Dicentrarchus labrax* L.). Moreover, we studied the synteny of the genomic locus and neighbouring genes with other selected fish species, the exon-intron organization of the two genes, and their basal expression in different organs and tissues. Modulation of the sea bass IL-2 and IL-2L genes was next studied, *in vitro* after stimulation with PHA and *in vivo* after *Vibrio* vaccination. Finally, the biological activity of the two IL-2 isoforms was investigated.

2. MATERIALS AND METHODS

2.1Cloning and sequence analysis of two sea bass IL-2 paralogs

Two nucleotide sequences related to different IL-2 isoforms, IL-2 and IL-2-like (IL-2L) (accession numbers KJ818330 and MF599338, respectively) were identified after the analysis of a sea bass (*Dicentrarchus labrax* L.) gill transcriptome [24]. The sequences have been confirmed by PCR cloning of the entire coding region (data not shown) from a sea bass gill cDNA obtained as described previously [25]. The sea bass IL-2 amino acid (aa) sequences were compared to each other and to counterparts in other species with the EMBOSS Pairwise Alignment tool. The IL-2 sequences were analysed for the presence of a signal peptide using SignalP software [26], and for N-linked glycosylation sites, with the NetNGlyc 1.0 Server. A multiple sequence alignment of the sea bass IL-2 aa sequences with selected IL-2 molecules from other species was carried out with the CLUSTALW algorithm included in the MEGA 7.0 software [27]. A phylogenetic tree was constructed using the multiple sequence alignment as an input for a neighbour-joining method-based clustering, carried out within MEGA 7. The evolutionary distances were computed using the JTT matrix-based method, with all ambiguous positions removed for each sequence pair.

2.2 Synteny and gene organization analysis of sea bass IL-2 and IL-2L

The IL-2 and IL-2L genes of sea bass *Dicentrarchus labrax* were manually re-annotated, based on the alignment between the full-length mRNA sequences, obtained as described above and the genomic DNA sequence, with MUSCLE [28]. Donor and acceptor splicing sites were subsequently refined with gene boundaries with NNSPLICE v. 0.9 [29]. The sea bass genome sequence was obtained from NCBI Genomes (GCA_000689215.1, seabass_V1.0) [30].

The publicly available genomes of teleost fish were obtained from Ensembl (release 95). The position of IL-2 and IL-2L genes was identified through a tBLASTn approach (with e-value threshold set to $1E^{-5}$) and neighbouring genes annotated at the 5' and 3' ends of the locus were inspected. Finally, their order and orientation were compared with the genome architecture found in sea bass.

The presence of conserved regulatory motifs shared by the two genes was searched in the 1Kb-long region located upstream of both gene transcription start sites with MEME v.5.0.4 [31]. The motifs identified on the same strand and similar position were checked for overlaps together with the detected possible transcription factor-binding sites using AliBaba 2.1 [32], searching for significant matches was made using the TRASFAC 4.0 database.

2.3 Analysis of the basal expression of sea bass IL-2 and IL-2L transcripts

To investigate the basal expression levels of sea bass IL-2 and IL-2L, four sea bass juveniles weighing ~100g were sampled and different tissues (muscle, liver, spleen, head kidney (HK), thymus, gills, peripheral blood leukocytes (PBL), gut, brain) were obtained as described before [25]. Total RNA was isolated from each tissue separately with TRIsure (Bioline), resuspended in DEPC treated water and used for reverse-transcription real-time quantitative PCR without pooling the tissue samples coming from the different fish. Controls for the presence of DNA contamination were performed using β-actin primers that bracket an intron (see Table I). For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used with the protocol described previously [33]. The expression level of the two IL-2 transcripts was determined with a Mx3000P real-time PCR system (Stratagene) as described before [33]. Specific PCR primers were designed for the amplification of about 150-200 bp-long products from the two IL-2 sequences (see Table I). A relative quantitation was performed, comparing the levels of the target transcripts (IL-2 and IL-2L) to a reference tissue (calibrator, in this case one of the muscle samples). A normalizer target (18S ribosomal RNA) was included to correct for differences in total cDNA input between samples. The results are expressed as the mean + SD of the results obtained from the four fish, with duplicate samples performed for each animal.

Table I.

2.4 In vitro and in vivo sea bass IL-2 and IL-2L expression analysis

The *in vitro* IL-2 and IL-2L expression was studied after stimulation of HK leukocytes isolated from four sea bass juveniles as described above. Cells were cultured in L-15 medium (Gibco) containing 10% FCS, adjusted to a concentration of $1X10^5$ cells/ml and incubated at 22° C with 10 µg/ml of leucoagglutinin from *Phaseolus vulgaris* (PHA, Sigma). The samples used as control were incubated with L-15 medium alone. To be sure that the obtained effect was due to the specific bioactivity of the recombinant proteins we added two more controls made after pre-heating the molecules for 20 minutes at 95 °C.

Total RNA was isolated with TRIsure (Bioline) as described above and used for real-time quantitative PCR without pooling the samples coming from the different fish. The primers and the real-time PCR conditions were the same as described above, except that the calibrator for this experiment was one of the time 0 controls. The results were expressed as the mean + SD of the results obtained from four fish and the differences with the time-

matched control were considered significant when $p<0.05$, using two-way ANOVA analysis followed by the Bonferroni's post-hoc test.

The *in vivo* IL-2 and IL-2L expression analysis was performed from fish vaccinated with a commercial vaccine developed against the sea bass bacterial pathogen *Vibrio anguillarum* (Aquavac Vibrio Oral, Merck) [34]. In brief, vaccination was performed on 50 sea bass individuals (weighing 30-40 g). The fish were fed at a feeding rate of 1.5 % of fish biomass per day with a commercial pellet diet (Skretting) supplemented over 10 days with Aquavac Vibrio Oral (the delivery was performed over 15 days with 5 days off in the middle, to achieve the final concentration of antigen recommended by the manufacturer). The control group of 50 size matched sea bass individuals was fed with the commercial diet only. For gene expression analyses, 7 fish/group/time point (0, 6, 24 h and 72 h after the end of the vaccination procedure) had their HK sampled. RNA extraction, cDNA preparation and real time PCR analyses were performed as described above using as calibrator one of the time 0 control samples.

2.5 Production of recombinant sea bass IL-2 and IL-2L

The nucleic acid sequences encoding for the sea bass mature IL-2 and IL-2L peptides were codon optimised using GENEius program and synthesized by Eurofins (Germany), and cloned into a pTri-Ex6 expression vector (Novagen) as described previously [35]. The recombinant constructs pTri-sIL-2 and pTri-sIL-2L encode a His-tag (MAHHHHHHHHG) followed by the mature peptide. Thus, the recombinant IL-2 and IL-2L proteins were 136 aa and 128 aa long, with a theoretical pI of 4.76 and 5.83, and a calculated molecular weight of 15.3 kDa and 14.7 kDa, respectively. A sequence confirmed plasmid was used to express the encoded proteins in BL21 Star (DE3) (Invitrogen). The recombinant proteins were induced by the auto-induction medium, purified under denaturing conditions, refolded, re-purified under native conditions, analysed and quantified on SDS-PAGE as described previously [18, 23]. Briefly, the recombinant proteins produced in *E. coli* were first dissolved in lysis buffer (50 mM Tris-HCl, pH8.0, 6 M GuHCl,500 mMNaCl,20 mM, 2-mercaptoethanol). The lysate was then sonicated and cleared by centrifugation (13,000 rpm, 30 min) and loaded onto a His GraviTrap column (GE Healthcare). Contaminants were removed by extensive washing with a wash buffer (lysis buffer supplemented with 20 mM imidazole and 1% Triton X-100) and the recombinant protein was eluted in elution buffer (lysis buffer with 500 mM imidazole). The resultant denatured pure protein was refolded in a refolding buffer (PBS, pH 7.2 supplemented with 10% glycerol, 0.5 M arginine monohydrochloride, 0.2% PEG 3350, and

5 mM 2-mercaptoethanol) for 2 days at 4 ℃. The purified proteins were finally desalted in desalting buffer (DSB, PBS with 10 mM arginine, 50% glycerol, and 5 mM 2-ME) using PD-10 Desalting Columns (GE Healthcare). After sterilization with a 0.2-µm filter, the recombinant proteins were aliquoted and stored at −80°C ready for stimulation of cells. The endotoxin (EU) level in the recombinant proteins was determined with a *Limulus* Amebocyte Lysate Kit (LAL Test, Bio Whittaker) as previously described [36].

2.6 In vitro biological activity of recombinant sea bass IL-2 and IL-2L

The *in vitro* biological activity of the recombinant IL-2 and IL-2L was studied using leukocytes isolated from HK and spleen of four sea bass juveniles (100 g of weight). Cells were cultured in L-15 medium (Gibco) containing 10% FCS, adjusted to $1X10^5$ cells/ml and incubated at 22° C for 4 h and 24 h with 200 ng/ml of each recombinant cytokine separately. The cell control samples were stimulated with DSB buffer alone.

Total RNA was isolated with TRIsure (Bioline) as described above and used for real-time quantitative PCR without pooling the samples coming from different fish. The biological activity of the recombinant IL-2 and IL-2L was monitored by studying the regulation of the transcript level of different target genes shown to be modulated in salmonids by IL-2 cytokines [23] and identified from the sea bass gill transcriptome. These included: IL-2, IL-2L, IL-4/13A1, IL-4/13A2, IL-4/13B, IFN-γ, TNF-α, IL-22, CD4 and IL-10. Specific PCR primers (see Table I) were designed for the amplification of products (~150-200 bp) from the conserved region of all selected genes. Real-time PCR conditions were the same as described above; the calibrator for this experiment was one of the time 0 controls that were freshly prepared before stimulation. The results were expressed as the mean + SD from four fish and the differences from the time-matched control were considered significant when $p<0.05$ using the two-way ANOVA analysis followed by the Bonferroni's post-hoc test.

2.7. In vitro proliferation effect of recombinant sea bass IL-2 and IL-2L

Three sea bass specimens were lethally anaesthetized with ethylene glycol monophenyl ether (Merck, Whitehouse Station, USA). Head kidney (HK) and spleen (SPL) were removed and immersed in cold Hanks Balanced Salt Solution without calcium and magnesium (HBSS), previously adjusted for appropriate osmolarity (355 mOsm Kg-1) with 3 M NaCl. Leukocytes were obtained by subsequently filtering the organs in cold HBSS through a 100 µm and 40 µm nylon mesh strainers with syringe pestles. The obtained cells

were washed by centrifugation (10 min, 400 g, 4 °C), resuspended in HBSS and layered over a discontinuous Percoll gradient at densities of 1.02 and 1.07 g cm-3 [25]. After centrifugation (30 min, 840 g, 4°C with no acceleration and deceleration), leukocytes at the interface between two densities were collected, washed with HBSS (10 min at 400 g, 4 °C), and resuspended in 5 ml of Leibovitz's L-15 medium (Sigma-Aldrich). Cells were counted with a Neubauer chamber.

Leukocytes from head kidney and spleen for each of the three biological replicates were adjusted to a concentration of 4 x 10^5 cells/ml in Leibovitz's L-15 medium (Sigma-Aldrich) and cultured in 96-well plates in presence of 1 µg/ml of rIL-2 alone, of rIL-2L alone and of a mixture rIL-2 and rIL-2L. A control consisting of untreated leukocytes was also included in the assay. Two technical replicates were considered for each experimental group. Plates were incubated at 22°C for 72 hours. Intracellular ATP as a proxy of proliferation was then semi-quantitatively evaluated using the ATPlite assay (PerkinElmer, Groningen, The Netherlands) following manufacturer's instructions. Briefly, 50 µl of cell lysis and substrate solutions were added to 100 µl of cell suspensions in each well and stirred for 5 minutes; plates were then dark-adapted for ten minutes and luminescence measured using a microplate reader (Wallac Victor2, PerkinElmer). Raw technical replicate readings were first employed in a Shapiro-Wilk normality test. Because they fulfilled parametric conditions, the 1-way ANOVA test followed by the post-hoc Tukey's multiple comparison test was used. Luminescence readings were then averaged within each biological replicate and a mean proliferation index (PI) was calculated as luminescence of stimulated cells divided by that of untreated samples for a more informative graphical representation.

2.8 Statistical analysis

The data of all real-time PCR experiments were expressed as the mean ± SD of the results obtained from four fish. The statistical analysis was performed using the software GraphPad Prism 4 (two-way ANOVA) and Sigma Plot (Bonferroni test). Data were considered significant when $p < 0.05$.

2.9 Use of experimental animals

All fishes were handled complying with the Guidelines of the European Union Council and of the Ethical Committee of Tuscia University for the use of live laboratory animals. All experiments were performed in accordance with relevant guidelines and regulations of the Ethical Committee.

3. RESULTS

3.1 The nucleotide sequence analysis of sea bass IL-2 and IL-2L

Two nucleotide sequences (IL-2 and IL-2L) related to a possible IL-2 cytokine have been identified in sea bass (accession numbers KJ818330 for IL-2 and MF599338 for IL-2L). The sea bass IL-2 sequence had an in frame stop codon, located in the 5' UTR, before the main open reading frame (ORF), that was absent in the IL-2L 5' UTR. The transcripts encoded two putative proteins of 145 and 137 aa for IL-2 and IL-2L respectively, with predicted signal peptides of 20 aa and 2 potential N-glycosylation sites in both (see Figures S1 and S2). The IL-2 sequence showed a potential polyadenylation signal 13 bp upstream of the polyA tail that was absent in the IL-2L sequence, but both had multiple ATTTA motifs in the 3'-UTR (see Figures S1 and S2).

3.2 The genomic structure and synteny analysis of sea bass IL-2 and IL-2L genes

The *Dicentrarchus labrax* IL-2 and IL-2L genes are located on the LG2 genomic super scaffold, with the same strand orientation, close to each other and separated by just ~3.5 Kb intergenic sequence. This situation mirrors the previously reported organization of IL-2 and IL-2L genes in stickleback and Fugu [23]. Consistent with previous reports from other teleost species, and in line with the genomic organization of mammalian IL-2 genes [14-15], both sea bass genes consisted of four exons and three introns, as shown in Figure 1.

Overall, the IL-2 gene is larger than the IL-2L gene (1,712 vs 1,042 base pairs), mainly due to a much longer 3'UTR region, and a longer intron 2 (144, 207 and 104 base pairs in introns of IL-2L; 94, 400 and 105 base pairs in introns of IL-2). The exon sizes (187, 38, 131 and 222 base pairs for IL-2L; 220, 38, 143 and 703 for IL-2) are quite comparable except for exon 4. Although the regulatory regions upstream to the transcription start site (TSS) of the two genes share little conservation, two conserved motifs were identified in a similar position. The first one, a 10-base pair long motif with consensus sequence CSCTCTGYGCC, located in position -179 in IL-2 and -186 in IL-2L, was identified as a putative binding site for the Sp-1 transcription factor. This conserved binding motif, known as ZIP, is found in a slightly different position in the promoter of mammalian IL-2 genes, where it is recognized by both Sp-1 (constitutively expressed) and by the inducible factor EGR-1 [37]. The second conserved motif is more distal, located ~800 base pairs upstream of the TSS in both genes (203 in IL-2, 205 in IL-2L). The consensus sequence (GGGGAAWCC) indicates that it may be recognized by NF-κB-family transcription factors.

Despite the relatively long distance of this element from the TSS, its presence is reminiscent of the binding site described by other authors in mammalian IL-2 promoters [38-39].

The genomic region which contains the IL-2 and IL-2L genes in *Dicentrarchus labrax* displays high synteny with homologous regions from other teleosts. This ~400 Kb-long genomic region, located on the sea bass genetic linkage group 2, includes 12 genes found in the same relative order and orientation in the majority of Euteleostei. This synteny block includes SPRY, SPATA5, NUDT6, FGF2, BBS12, CETN4 and IL-21 upstream, and ADAD1, TLX2, NPM1B, FGF24, PCGF1 and LBX2 downstream of the IL-2L/IL-2 genes (Figure 1), as previously reported by other authors [23]. Not considering teleost species which underwent a 4R WGD, this synteny block was found in 30 out of the 31 Euteleost genomes deposited in Ensembl (release 95), except for a few lineage-specific gene losses (e.g. SPRY1 in stickleback).

This synteny block was broken in the most basal fish lineages, such as Otocephala (e.g. Cypriniformes, Characiformes and Siluriformes), Osteoglossomorpha (e.g. Asian bonytongue) and in the Holostean Lepisosteiformes (e.g. spotted gar). Indeed, these species show a different set of genes downstream of ADAD-1 as seen in spotted gar (Figure 1). Interestingly, the organization of the genes flanking ADAD-1 in spotted gar, the most basal bony fish species with a sequenced genome (KIAA1109, TRPC3, BBS7, CCNA2 and EXOSC9) is identical to human. Overall, these results indicate that the general architecture of the genomic region where IL-2 and its paralogous genes evolved has remained nearly invariable for the past 400 million years of animal evolution.

Figure 1.

3.3 The amino acid sequence analysis of sea bass IL-2 and IL-2L

The amino acid sequence identity and similarity of sea bass IL-2 and IL-2L with other known IL-2 molecules is shown in Table II. For sea bass IL-2, the highest aa identity was found with IL-2 from three-spined stickleback (39.3%), followed by yellow croaker (38.3%) and Fugu (38.3%). Similar results were obtained for sea bass IL-2L that showed highest aa identity with three-spined stickleback (39.0%), followed by Fugu (35.0%). Overall, the identity values are quite low, even between sea bass IL-2 and IL-2L (only 32.9%). A multiple sequence alignment with different fish IL-2 and percomorph IL-2L cytokines, together with human IL-2 was assembled (see Figure 2, A and B). Although six cysteine residues are present in both sea bass IL-2 and IL-2L mature proteins, this number is quite variable in the

different teleost IL-2 molecules. However, two cysteine residues, involved in a disulphide bond (Cys78 and Cys125, see PDB: 1M47) within the crystal structure of human IL-2 [40], are conserved in all percomorph IL-2 sequences (see Figure 2A). The mammalian IL-2 family signature (TELKHLQCLEEEL) found in the PROSITE database [41] could also be found in sea bass IL-2 and, within this region, the residues Leu63, Cys65 and Glu69 are conserved. The possible presence of four α helices was revealed in both sea bass IL-2 and IL-2L using the analysis within the PredictProtein website (https://www.predictprotein.org/), even though the first one (α helix A) in sea bass IL-2L is very short compared to human IL-2. Finally, Tyr65 and Glu82, which have been shown to be the most energetically critical residues in the IL-2Rα/IL-2 interface in human IL-2 [42], are conserved in most teleost IL-2 sequences and, therefore, it can be speculated they might also be important in the interactions between fish IL-2 and its receptor, despite the IL-2Rα being absent in fish genomes.

In the phylogenetic tree analysis (Figure 3) the sea bass IL-2 and IL-2L molecules cluster with all other fish IL-2 sequences. The known protacanthopterygian (i.e. salmon/trout) and otocephalan sequences (i.e. carp) sequences form a separate clade containing both sequences identified as IL-2A and IL-2B, which have been originated by lineage-specific 4R WGD events. All other considered teleost IL-2L sequences are present in a separate branch with high bootstrap support (99%).

Table II. Figure 2 and Figure 3.

3.4 Basal expression of sea bass IL-2 and IL-2L

The constitutive expression of sea bass IL-2 and IL-2L has been analysed in 9 different tissues of four healthy fish (see Figure 4A and B). Both IL-2 and IL-2L are highly expressed in gut, followed, at lower levels, by brain. Low transcript levels were found in liver and gills for both cytokines.

Figure 4.

3.5 Expression analyses of sea bass IL-2 and IL-2L in vitro and in vivo

To investigate the modulation of expression of sea bass IL-2 and IL-2L, we performed an *in vitro* stimulation of leukocytes from HK with PHA, a T cell mitogen (Figure 5A). PHA stimulated a significant increase of both IL-2 and IL-2L expression at 4h and 24h. Moreover,

IL-2L was significatively more highly up-regulated at the same time points compared to IL-2 ($p < 0.05$).

To investigate the involvement of IL-2 and IL-2L in the sea bass immune response following vaccination, we evaluated their gene expression after administration of a commercial oral vaccine against *Vibrio anguillarum*, which is commonly used in aquaculture. A statistically significant up-regulation of both IL-2 and IL-2L was found in HK leukocytes at 6h (Figure 5B) after vaccination, and again IL-2L was more highly induced ($p < 0.05$).

Figure 5.

3.6 Production of the recombinant sea bass IL-2 and IL-2L

Single proteins for sea bass recombinant IL-2 (rIL-2) and rIL-2L, at the calculated molecular weight of 15.3 kDa and 14.7 kDa respectively, have been purified after refolding (see Figure S3); denaturants and other contaminants were removed by extensive washing of the purification column. The bacterial endotoxin contamination of the purified proteins was determined and shown to be less than 10 ng/1μg of protein (31.25 EU/μg of protein); therefore, at the concentration used to study the biological activity of the recombinant cytokines (200 ng/ml) the LPS concentration did not exceed 10 pg/ml, which is considerably less than the minimum amount needed to induce pro-inflammatory genes in fish HK leukocytes [43].

3.7 In vitro biological activity of recombinant sea bass IL-2 and IL-2L

The biological activity of the sea bass rIL-2 and rIL-2L isoforms on HK and spleen leukocytes has been investigated (Figures 6, 7, 8 and 9) after 4 h and 24 h stimulation, using the same concentration previously selected for trout rIL-2 isoforms on PBL (Wang et al., 2018). The effects of the rIL-2 and rIL-2L on sea bass leukocytes were studied in relation to the expression of several important immune-related genes, including: IL-2 and IL-2L itself (Figure 6), IFN-γ and TNF-α (Th1 pathway, Figure 7), IL-4/13A1, IL-4/13A2 and IL-4/13B (Th2 pathway, Figure 8), IL-22 (Th17 pathway), IL-10 (regulatory pathway), and CD4 (T cell marker) (Figure 9). The rIL-2L protein significantly up-regulated the expression of both IL-2 and IL-2L in head kidney at 24 h (Figure 6 A). However, in spleen no effect has been recorded for either recombinant (Figure 6 B). IFN-γ and TNF-α are fundamental cytokines secreted by Th1 cells [5] and, therefore, we decided to investigate if sea bass rIL-2 and rIL-

2L could modulate the expression of their transcripts. Our results showed that rIL-2L up-regulated both cytokines in a significant manner (Figure 7 A) in head kidney at 24 h, whilst rIL-2 weakly up-regulated only TNF-α at 24 h. In contrast, in spleen we found that rIL-2 was able to induce a significant up-regulation of IFN-γ and TNF-α after 24 h (Figure 7 B). Next, we investigated the modulation of expression of the Th2 pathway related cytokines IL-4/13A1, IL-4/13A2 and IL-4/13B. In head kidney (Figure 8 A), sea bass rIL-2L significantly up-regulated the expression of all three IL-4/13 isoforms after 24 h of stimulation, whereas rIL-2 produced a significant increase of only IL-4/13B expression at the same time point. In spleen (Figure 8 B), rIL-2L significantly up-regulated all IL-4/13 isoforms after 24 h, as in head kidney, but here rIL-2 was effective at increasing both IL-4/13A1 and IL-4/13B, albeit at a lesser extent compared to rIL-2L. Finally, we studied the modulation of IL-22, as a cytokine involved in the Th17 pathway. This transcript was significantly up-regulated at 24 h only in head kidney (Figure 9 A) by both recombinants, although rIL-2L had the largest effect. IL-10, as a regulatory cytokine, was also significantly up-regulated in only head kidney after 24 h of stimulation by both recombinants (Figure 9 A). Interestingly, CD4, a specific Th-cell marker, was significantly up-regulated in only spleen after 24 h stimulation by both rIL-2 and rIL-2L (Figure 9 B). The gene modulation due to the addition of the two IL-2 paralogs on the cell cultures was practically erased by their pre-heating and this confirms that the effect is specifically due to the biological activity of these recombinant cytokines (data not shown).

Figure 6-9.

3.8 In vitro proliferation effect of recombinant sea bass IL-2 and IL-2L

To investigate the activity of the two recombinant IL-2 isoforms as lymphocyte growth factors, we performed a proliferation assay on head kidney and spleen derived leukocytes stimulated with rIL-2, rIL-2 and rIL-2+rIL-2L. A significant increase of cell number was evidenced in both tissues after 72 h of incubation, although at a slight higher level in spleen (Figure 10, Panel A and B). The mixture of both cytokines does not seem to induce an additive effect.

Figure 10.

4. DISCUSSION

In mammals IL-2 is a fundamental immunomodulatory cytokine, secreted mainly by T-helper cells, and involved primarily in the proliferation, activation and differentiation of T cells. In this paper, we have identified two IL-2 isoforms from sea bass (*Dicentrarchus labrax* L.) and have undertaken a preliminarily characterization of them both from a structural and functional point of view.

We first explored the site where these two genes are present on the sea bass genome and determined that they are located at the same genomic site (see Figure 1), with the clear indication that the presence of these two IL-2 paralogs is due to a local gene duplication event. In salmonids, where two IL-2 paralogs also exist, synteny analysis showed that the IL-2A and IL-2B genes arose from the 4R salmonid whole genome duplication event [23], which took place approximately 95 Mya [44]. Hence the origin of the duplicated IL-2 genes is fundamentally different in these diverse fish lineages. Moreover, sea bass IL-2 and IL-2L share only low aa sequence identity (see Table II) as they do compared with the single IL-2 cytokine gene present in humans and with IL-2 paralogs from other fish species. This feature is likely related to the observation that cytokine genes are amongst the most rapidly evolving genes [45], probably as they are under diversification selection due to host-parasite co-evolution [14].

Considering the presence in sea bass of these two highly divergent IL-2 paralogs, we decided to explore the possibility that they have also undergone sub-functionalisation, as recently investigated in salmonids [23]. We started by comparison of the gene organization of these two IL-2 isoforms. Both genes have a four exons organization, but the IL-2 gene is larger than IL-2L, primarily due to a longer 3'UTR, intron 2 and exon 4 compared to IL-2L. Furthermore, the upstream regulatory regions of the two genes were quite divergent, suggesting variation in the control of gene expression. Differences in the size of exons have also been seen for IL-2A and IL-2B in salmonids, even though (as mentioned above), these paralogs arose in a different way compared to the IL-2 genes found in percomorphs [23]. Next, we analysed the primary structure of the two sea bass IL-2 cytokines. Both mature sequences show the presence of 6 cysteine residues which could possibly form three disulphide bonds; this possibility was confirmed using the DISULFIND prediction server (data not shown) [46]. Six cysteine residues are also present in stickleback and Fugu [14], whereas in salmonids IL-2A has three predicted disulphide bonds and IL-2B only two [23]. A single disulphide bond, fundamental for the stabilization of the biological active structure, occurs in human IL-2 [47]. In chicken IL-2, an additional pair of cysteine residues (Cys63

and Cys116) is present and may be involved in the formation of a disulphide bond [48]: this feature is conserved in most teleost IL-2 molecules. However, the hypothesis that more disulphide bonds are needed to stabilize the IL-2 and IL-2L structure in sea bass needs to be confirmed by further analyses. Exon 4 codes for helix C and D in the human protein, with helix C involved in assembly of the high-affinity trimeric receptor (IL-2Rα/IL-2Rβ/γC) that is fundamental for cell signalling [49]. Hence, the different sizes of this region in sea bass IL-2 and IL-2L could possibly reflect differences in the interaction with a single receptor or even that two different receptor complexes are present. The pI of the IL-2 and IL-2L proteins is acidic, in agreement with the result obtained for IL-2A in salmonids and IL-2 in other percomorphs, while IL-2B in salmonids shows a higher pI value [23].

The two IL-2 sea bass cytokines are expressed constitutively at high level in the same tissues (gut and brain). The expression of the IL-2 isoforms in brain could reflect the involvement of this cytokine in specific neurological processes as it happens in mammals [50]; moreover, it should be taken into account that both in green spotted pufferfish and in orange-spotted grouper there is an high expression of interleukin-2 enhancer binding factor (ILF2), a molecule that regulates IL-2 expression in mammals, in brain [51, 52]. This pattern is quite variable in the fish examined to date. For example, in rainbow trout the highest expression of IL-2A and IL-2B is in thymus and spleen [23], in Fugu no IL-2 expression was found in unstimulated fish [14], while in yellow croaker spleen and blood were the sites with the highest level of IL-2 transcripts [19]. Sea bass IL-2 and IL-2L are up-regulated after stimulation of HK leukocytes with the T cell mitogen PHA, at both 4 h and 24 h after treatment, although IL-2L transcript levels were more highly induced. This agrees with results for IL-2 from Fugu [14] and yellow croaker [19], and for IL-2A and IL-2B from rainbow trout, where IL-2B is more inducible compared to IL-2A [23]. Moreover, the sea bass IL-2 paralogs are up-regulated in HK leukocytes 6 h after oral vaccination with a bacterin of the fish pathogen *V. anguillarum*, where again IL-2L showed a higher increase. These samples were already analysed in a previous paper and other immune-markers, like IL-4/13 isoforms, IL-1β and IL-10 (data not shown), are modulated by the vaccination [34]. These data agrees with the up-regulation of IL-2 in yellow croaker after stimulation of spleen and HK cells with a trivalent bacterial vaccine [19]. The differences in inducibility seen with these stimulants may reflect the divergent upstream regulatory regions for IL-2 and IL-2L.

Finally, a preliminary examination of the functional activity of sea bass IL-2 and IL-2L was undertaken to compare their activity for the first in percomorphs. We produced the

two recombinant cytokines in *E. coli* and then investigated their effect *in vitro* on HK and spleen leukocytes. In mammals, both effector Th cell subsets (Th1, Th2, Th17) and T-regulatory cells have been identified and each cell type expresses a specific set of cytokines [53]. In sea bass, most of these molecules are present and, therefore, we studied whether both IL-2 paralogs could up-regulate signature cytokines for Th1 cells (IL-2, IL-2L, IFN-γ, TNF-α), Th2 cells (IL-4/13A1, IL-4/13A2, IL-4/13B), Th17 cells (IL-22), Treg cells (IL-10) and Th cells more generally (CD4). No significant changes were found 4h post-stimulation with the recombinant cytokines, but at 24 h we found that rIL-2L is capable of up-regulating IL-2, IL-2L, IFN-γ and TNF-α in HK cells, while rIL-2 could increase the transcript levels of IFN-γ and TNF-α in spleen. rIL-2L also up-regulates all the IL-4/13 isoforms in head kidney and spleen at this timing, whereas rIL-2 only induced IL-4/13B in HK cells and to some extent IL-4/13A1 and IL-4/13B in splenocytes. rIL-2 and rIL-2L both increased the expression of IL-22 and IL-10 in HK cells, but had no effect on spleen cells. In contrast, CD4 was up-regulated by both rIL-2 and rIL-2L but only in spleen. In yellow croaker rIL-2 up-regulates markers for Th1 and Th2 subsets in both the HK and spleen, but it has no effect on Th17 markers [19], with similar effects found for rainbow trout rIL-2A and rIL-2B, although a limited effect on Treg signature cytokines was seen [23]. These differences in responsiveness of the two IL-2 paralogs may reflect the presence of different cell populations (ie in the HK and spleen) in the examined tissues, or potentially the downstream effects of different signalling pathways as different receptors should bind IL-2 and IL-2L in sea bass. Both sea bass rIL-2 and rIL-2L promote *in vitro* HK and spleen derived leukocyte growth, although we do not have direct evidence that this is due specifically to T cell growth, as it happens for mammalian IL-2 [8]. The effect we see in sea bass is not additive using both cytokines in the same stimulation assay and this could probably be due to a saturation of the IL-2 cell surface receptors. In trout, rIL-2B treated PBL showed a significant increase of proliferation, while no effect was seen using rIL-2A [23].

5. CONCLUSIONS

In conclusion, two divergent IL-2 paralogs have been identified in sea bass. The sequences share 32.9% amino acid identity and should, therefore, possess some structural differences and, potentially even preferential binding to different sea bass IL-2 cell receptor(s). Both molecules were induced in sea bass after bacterial vaccination, and hence may promote T cell proliferation and immune responses *in vivo*, as in mammals. Whilst IL-2 and IL-2L have some shared functional activities, differences in bioactivity were seen. IL-2L

promotes expression of markers of Th1, Th2, Th17 and Treg cell subsets in HK cells, while in splenocytes this cytokine has effects on markers of Th2-type responses and Th cells themselves (CD4). IL-2 shows a more limited activity, mainly in spleen on both Th1 and T cell markers. Moreover, in *in vitro* assays both cytokines are able to promote cell proliferation in head kidney and spleen. Therefore, our results suggest that IL-2 and IL-2L have an important role in sea bass immune responses and may regulate T cell development and differentiation in this species.

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AUTHOR CONTRIBUTIONS

V.S., E.R., T.W., M.C.B. and M.G., P.R.S. and A.M. performed the experiments; C.J.S., A.P. and G.S. analysed the data. F.B., T.W. and C.J.S. conceived and designed the experiments. F.B., T.W., M.G. and C.J.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1. ***Schematic representation of the seabass IL-2/IL-2L locus and neighbouring genes in comparison to the gar (Lepisosteus aculeatus) and Amazon molly (Poecilia formosa) loci.*** In addition, the exon/intron organization of the two seabass genes is shown (gene annotations are indicated in blue, mRNA annotations are indicated in green, CDS annotations are indicated in yellow). The position of the two conserved potential Sp-1 and NF-κB binding sites is indicated. Accession numbers: NC_023182.1 (*Lepisosteus oculatus*); NW_006799978.1 (*Poecilia formosa*); CBXY010011116.1 (*Dicentrarchus labrax*).

Figure 2. ***A and B. Amino acid sequence alignment of the predicted sea bass IL-2 and IL-2L isoforms with selected IL-2 molecules***. The cysteine residues are highlighted in bold along the sequences, except for the ones present in the signal peptide. The amino acid residues conserved in all sequences are shown in bold and underlined. The four α-helices found using the PredictProtein website are showed above the alignments. The IL-2 family signature identified by PROSITE is underlined in the IL-2 and IL-2L sea bass amino acid sequences. The signal peptide is in italics in the sea bass IL-2 paralogs. The human IL-2 sequence has been added as a reference: within this sequence the four α-helices are evidenced in italics and the IL-2 family signature is underlined. For accession numbers see Table S1.

Figure 3. ***Phylogenetic tree analysis of teleost fish IL-2 and IL-2L molecules.*** The phylogenetic tree was constructed using amino acid multiple alignments and the neighbour-joining method within the MEGA4 program. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) was shown next to the branches. 0.2 represents the genetic distance. For accession numbers see Table S1.

Figure 4. ***Basal expression of sea bass IL-2 and IL-2L in different tissues.*** Sea bass IL-2 (A) and IL-2L (B) mRNA levels were first normalised to that of 18S rRNA in the same tissue after real-time PCR analysis and then expressed as arbitrary units using the average expression level in the liver (AU=1 unit). Data are presented as the mean + SD of four healthy sea bass juveniles. *= $p < 0.05$ and **= $p < 0.01$, ***= $p < 0.001$ with respect to the liver.

Figure 5. **Expression of s*ea bass IL-2 and IL-2L after in vitro stimulation with PHA and vaccination against Vibrio anguillarum.* A)**. The mRNA levels of sea bass IL-2 and IL-2L were normalised to that of 18SrRNA in the same samples after real-time PCR analysis of HK leukocytes stimulated with L-15 medium (control) or with 10 µg/ml of PHA for 4 h and 24 h and expressed as arbitrary units using the average expression in non-stimulated 0 h controls (AU=1 unit). Data are presented as the mean + SD. **= $p < 0.01$and ***= $p < 0.001$, with respect to the time 0 control; N=4. **B)** Sea bass IL-2 and IL-2L mRNA levels were expressed as a ratio relative to rRNA 18S in the same samples after real-time PCR analysis of HK leukocytes of four fish vaccinated against *Vibrio anguillarum*, and then expressed as arbitrary units using the average expression in non-stimulated 0 h controls (AU=1 unit). Data are presented as the mean + SD. *= $p < 0.05$, with respect to the time 0 control.

Figure 6. ***Biological activity of sea bass recombinant (r)IL-2 and rIL-2L.*** The expression level of genes coding for IL-2 and IL-2L was determined in HK and spleen leukocytes after stimulation with the sea bass rIL-2 and rIL-2L. Transcription values were expressed as a ratio

relative to 18S rRNA in the same samples and then expressed as arbitrary units using the average expression in non-stimulated 0 h controls (AU=1 unit). The quantitative PCR amplification was performed in PCR arrays and each point represents the mean + SD of cells from 4 individual fish. *= $p < 0.05$, with respect to the time matched control.

Figure 7. ***Biological activity of sea bass recombinant (r)IL-2 and rIL-2L.*** The expression level of genes coding for IFN-γ and TNF-α was determined in HK and spleen leukocytes after stimulation with the sea bass rIL-2 and rIL-2L. Transcription values were expressed as a ratio relative to 18S rRNA in the same samples and then expressed as arbitrary units using the average expression in non-stimulated 0 h controls (AU=1 unit). The quantitative PCR amplification was performed in PCR arrays and each point represents the mean + SD of cells from 4 individual fish. *= $p < 0.05$ and **= $p < 0.01$, with respect to the time matched control.

Figure 8. ***Biological activity of sea bass recombinant (r)IL-2 and rIL-2L.*** The expression level of genes coding for IL-4/13A1, IL-4/13A2 and IL-4/13B was determined in HK and spleen leukocytes after stimulation with the sea bass rIL-2 and rIL-2L. Transcription values were expressed as a ratio relative to 18S rRNA in the same samples and then expressed as arbitrary units using the average expression in non-stimulated 0 h controls (AU=1 unit). The quantitative PCR amplification was performed in PCR arrays and each point represents the mean + SD of cells from 4 individual fish. *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$, with respect to the time matched control.

Figure 9. *Biological activity of sea bass recombinant (r)IL-2 and rIL-2L.* The expression level of genes coding for IL-22, IL-10 and CD4 was determined in HK and spleen leukocytes after stimulation with the sea bass rIL-2 and rIL-2L. Transcription values were expressed as a ratio relative to 18S rRNA in the same samples and then expressed as arbitrary units using the average expression in non-stimulated 0 h controls (AU=1 unit). The quantitative PCR amplification was performed in PCR arrays and each point represents the mean + SD of cells from 4 individual fish. *= $p < 0.05$ and **= $p < 0.01$, with respect to the time matched control.

Figure 10. *In vitro* proliferation of HK and spleen leukocytes due to IL-2 and IL-2L. The proliferation effect of rIL-2, rIL-2L and rIL-2+rIL-2L addition to HK (Panel A) and spleen (Panel B) derived leukocytes has been analysed. The ATPlite assay has been performed on three individual fishes with 2 technical replicates. Data are presented as mean ± SD proliferation index, calculated as luminescence of IL-treated cells divided by the value obtained for untreated samples. *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$.

TABLE LEGENDS

Tab. I. Primers used for expression analysis.

Tab. II. Percentages of amino acid identity and similarity of sea bass IL-2 and IL-2L cytokines with other selected IL-2 sequences. The highest percentage values are highlighted in bold. For accession numbers see Table S1.

Table I.

Gene	Primers Sequence 5'-3'(forward, FW, and reverse, RV)	Accession number
β-actin	ATGTACGTTGCCATCC (FW) GAGATGCCACGCTCTC (RV)	AJ493428
18S ribosomal RNA	CCAACGAGCTGCTGACC (FW, real-time PCR) CCGTTACCCGTGGTCC (RV, real-time PCR)	AY831388
IL-2	GCTTTACTCAAGCACTGGAC (FW, real-time PCR) GCCATCGTTCAATCGTTGATC (RV, real-time PCR)	KJ818330
IL-2L	CGCAGCCATGGAGCGCTTCA (FW, real-time PCR) ATAGCACTCGTTTCCCACTTTC (RV, real-time PCR)	MF599338
IL-4/13A1	ATGGTGCAAACAAATGTCAGGATAA (FW, real-time PCR) TGACGTCTGAAGGGACCACAT (RV, real-time PCR)	KJ818332
IL-4/13A2	GCAGCAGAAAATGTGAGGATCG (FW, real-time PCR) GATCTCTATGCCTGTACTTGTGTCATTC (RV, real-time PCR)	KJ818333
IL-4/13B	TCATGAAGACGCAAATCTGATGT (FW, real-time PCR) CGAGACAGGAGAACTCTTTCACACA (RV, real-time PCR)	KJ818331
IFN-γ	TCAAGATGCTGAGGCAACAC (FW, real-time PCR) GAGCTTGCCTCCTCGTACAGC (RV, real-time PCR)	KJ818329
TNF-α	CGACTGGCGAACAACC (FW, real-time PCR) GCTGTCCTCCTGAGC (RV, real-time PCR)	DQ200910
IL-22	CACCGCCTGAAGACCGACC (FW, real-time PCR) GTGAACAGGATGTCGATCTCTCC (RV, real-time PCR)	KJ818327
CD4	GTGATAACGCTGAAGATCGAGCC (FW, real-time PCR) GAGGTGTGTCATCTTCCGTTG (RV, real-time PCR)	AM849811
IL-10	ACCCCGTTCGCTTGCCA (FW, real-time PCR) CATCTGGTGACATCACTC (RV, real-time PCR)	AM268529

Table II.

	Amino Acid Identity	***Amino Acid Identity***	***Amino Acid Similarity***	***Amino Acid Similarity***
	IL-2	**IL-2L**	**IL-2**	**IL-2L**
Dicentrarchus labrax IL-2		32.9		49.0
Dicentrarchus labrax IL-2L	32.9		49.0	
Salmo salar IL-2A	31.4	24.4	50.3	43.8
Oncorhynchus mykiss IL-2A	29.3	23.7	47.8	41.0
Oncorhynchus kisutch IL-2A	31.4	22.3	45.8	44.6
Oncorhynchus tshawytscha IL-2A	29.4	26.7	47.1	45.3
Salvelinus alpinus IL-2A	26.4	24.8	44.0	45.3
Oncorhynchus tshawytscha IL-2	24.8	20.1	44.4	32.5
Takifugu rubripes IL-2	38.3	25.2	53.2	40.9
Tetraodon nigrodiviris IL-2	37.0	27.6	49.3	48.0
Gasterosteus aculeatus IL-2	**39.3**	21.5	**54.0**	38.3
Cyprinus carpio IL-2A	28.7	20.8	46.5	43.0
Cyprinus carpio IL-2B	22.0	25.7	37.7	45.4
Oryzias latipes IL-2	31.0	22.4	46.8	44.7
Larimichthys crocea IL-2	38.3	25.0	53.5	37.5
Homo sapiens IL-2	15.0	19.5	23.3	32.9
Salmo salar IL-2B	28.2	22.1	46.2	36.9
Oncorhynchus mykiss IL-2B	24.2	20.1	44.4	31.8
Oncorhynchus kisutch IL-2B	24.4	21.8	42.3	38.5
Salvelinus alpinus IL-2B	25.0	21.5	42.8	32.9
Takifugu rubripes IL-2L	24.0	35.0	38.0	51.0
Tetraodon nigrodiviris IL-2L	17.8	28.2	28.9	45.8
Gasterosteus aculeatus IL-2L	29.8	**39.0**	41.8	**52.5**

Figure 1.

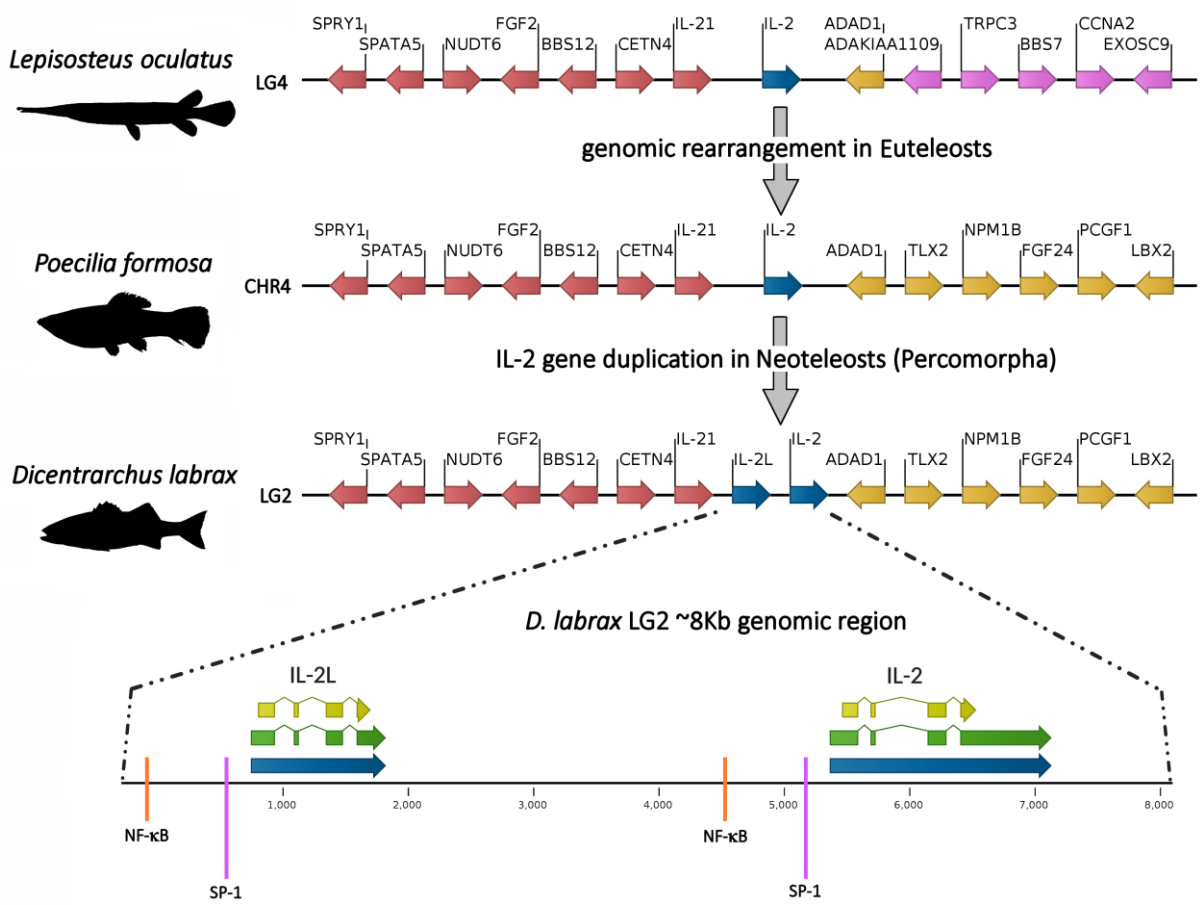

Lepisosteus oculatus
LG4
SPRY1
SPATA5
FGF2
NUDT6
BBS12
CETN4
IL-21
IL-2
ADAD1
ADAKIAA1109
TRPC3
BBS7
CCNA2
EXOSC9
genomic rearrangement in Euteleosts
Poecilia formosa
CHR4
SPRY1
SPATA5
FGF2
NUDT6
BBS12
CETN4
IL-21
IL-2
ADAD1
TLX2
NPM1B
FGF24
PCGF1
LBX2
IL-2 gene duplication in Neoteleosts (Percomorpha)
Dicentrarchus labrax
LG2
SPRY1
SPATA5
FGF2
NUDT6
BBS12
CETN4
IL-21
IL-2L
IL-2
ADAD1
TLX2
NPM1B
FGF24
PCGF1
LBX2
D. labrax LG2 ~8Kb genomic region
IL-2L
IL-2
1,000
2,000
3,000
4,000
5,000
6,000
7,000
8,000
NF-κB
SP-1
NF-κB
SP-1

Figure 2.

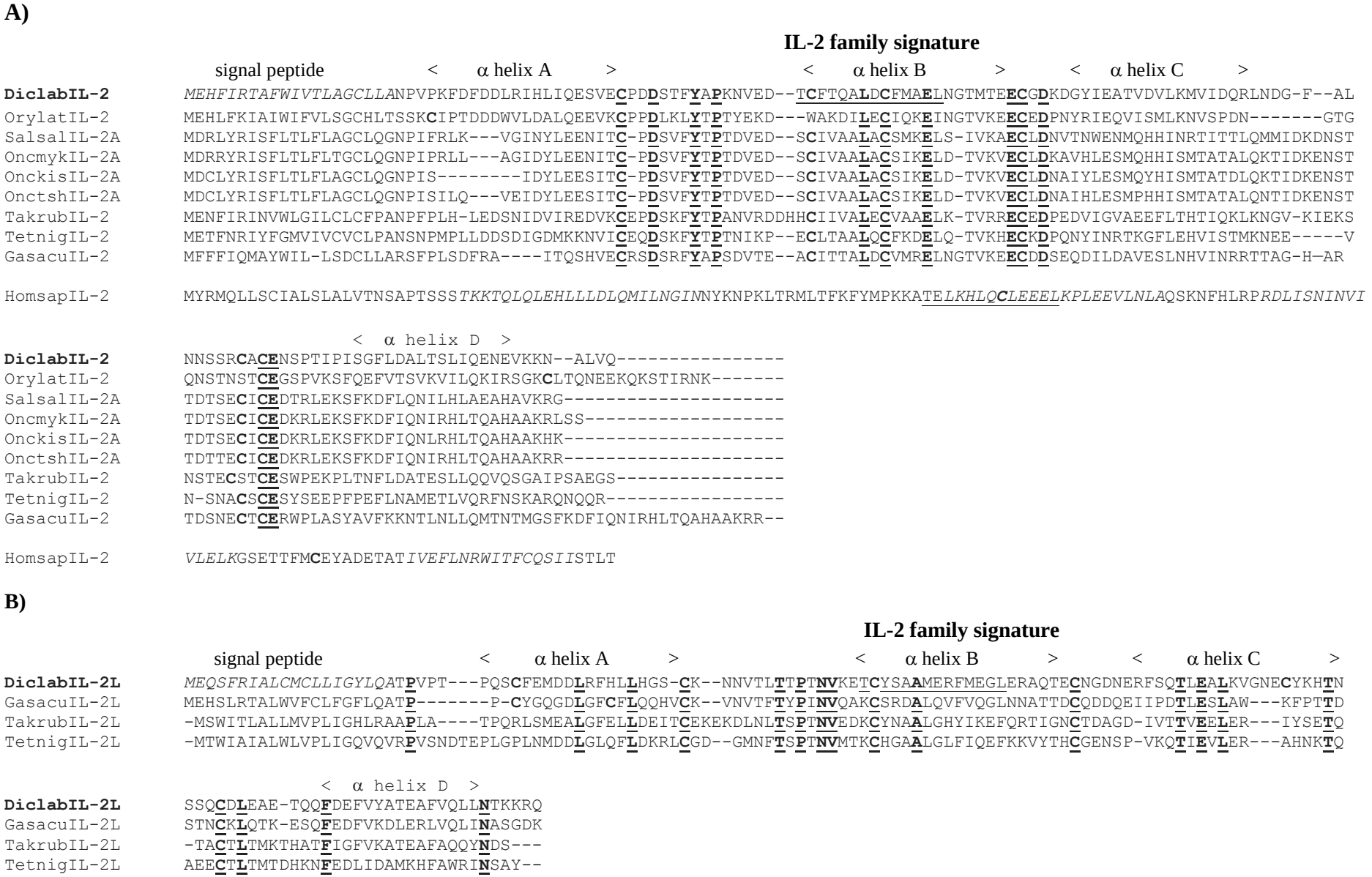

A)
IL-2 family signature
signal peptide
< α helix A >
< α helix B >
< α helix C >
DiclabIL-2
OrylatIL-2
SalsalIL-2A
OncmykIL-2A
OnckisIL-2A
OnctshIL-2A
TakrubIL-2
TetnigIL-2
GasacuIL-2
HomsapIL-2
< α helix D >
B)
IL-2 family signature
signal peptide
< α helix A >
< α helix B >
< α helix C >
DiclabIL-2L
GasacuIL-2L
TakrubIL-2L
TetnigIL-2L
< α helix D >

Figure 3.

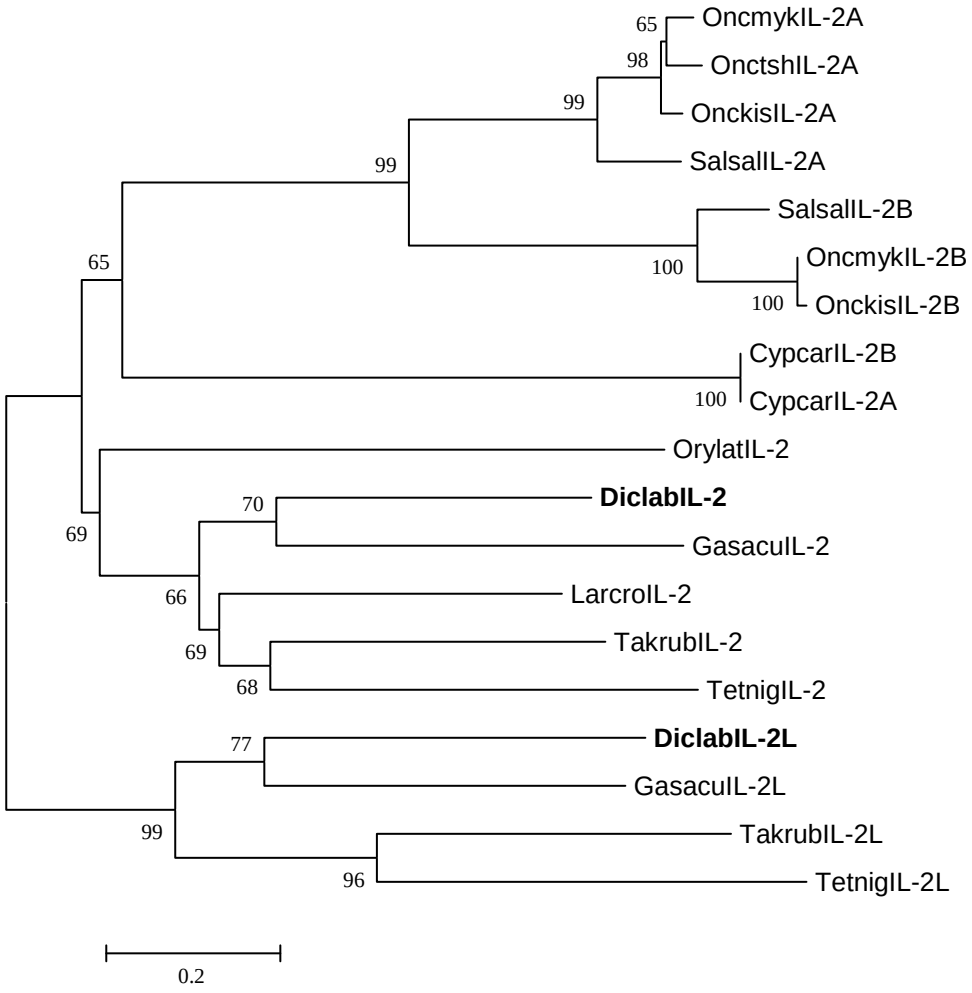
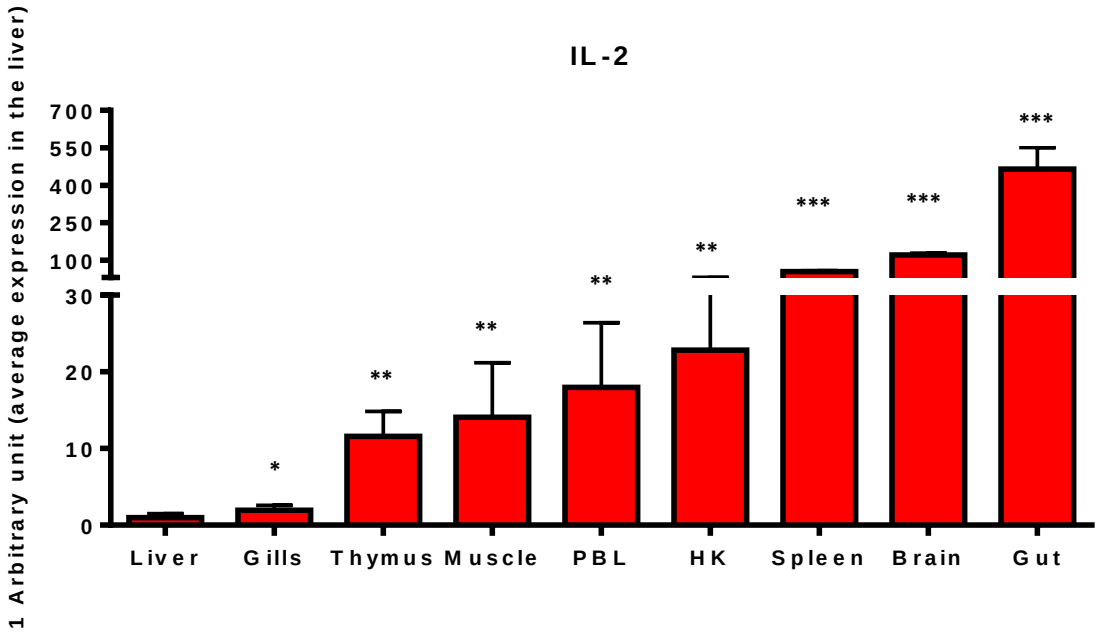

65
OncmykIL-2A
98
OnctshIL-2A
99
OnckisIL-2A
99
SalsalIL-2A
SalsalIL-2B
65
100
OncmykIL-2B
100
OnckisIL-2B
CypcarIL-2B
100
CypcarIL-2A
OrylatIL-2
70
DiclabIL-2
69
GasacuIL-2
66
LarcroIL-2
69
TakrubIL-2
68
TetnigIL-2
77
DiclabIL-2L
GasacuIL-2L
99
TakrubIL-2L
96
TetnigIL-2L
0.2

Figure 4.

A)


IL-2
1 Arbitrary unit (average expression in the liver)
700
550
400
250
100
30
20
10
0
*
**
**
**
**
***
***
***
Liver
Gills
Thymus
Muscle
PBL
HK
Spleen
Brain
Gut

B)

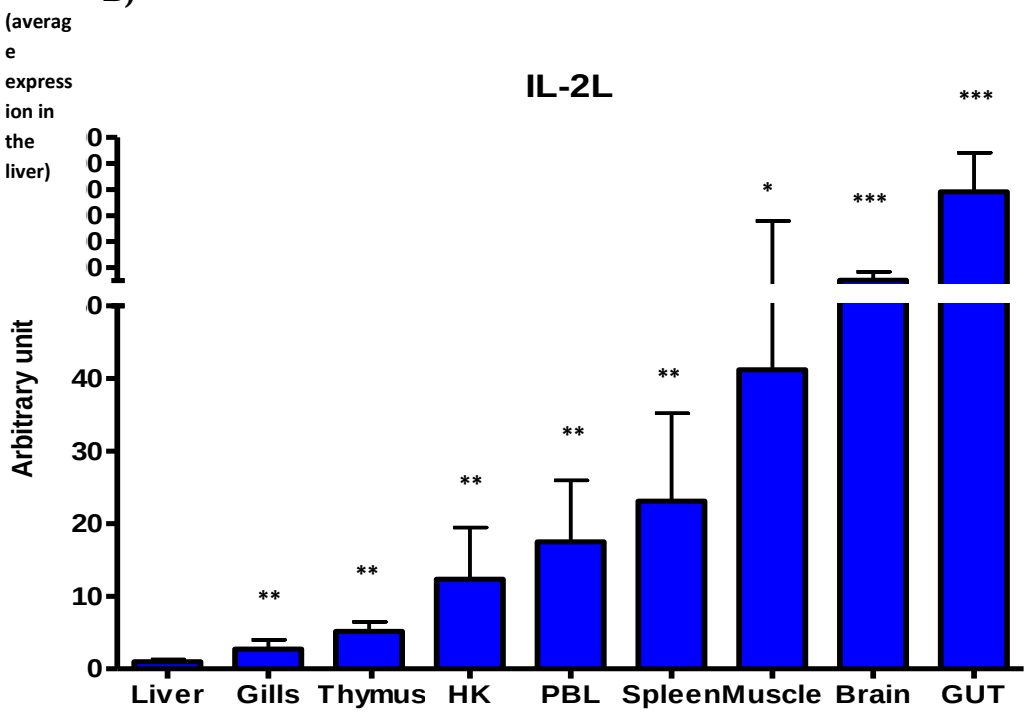
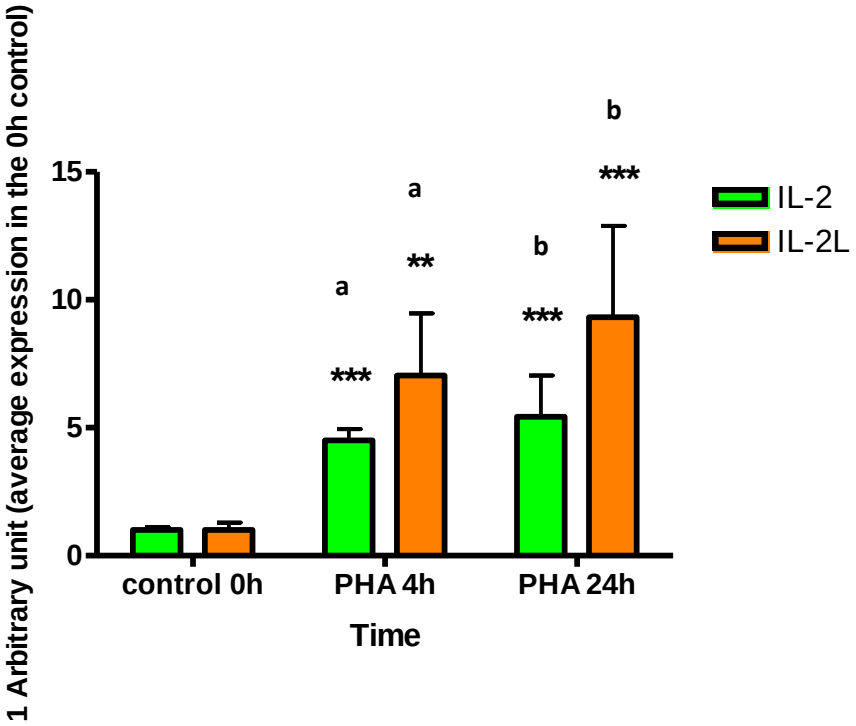

IL-2L
(average expression in the liver)
Arbitrary unit
0
0
0
0
0
0
40
30
20
10
0
**
**
**
**
**
*
***
***
Liver
Gills
Thymus
HK
PBL
Spleen
Muscle
Brain
GUT

Figure. 5.

A)


1 Arbitrary unit (average expression in the 0h control)
15
10
5
0
a
***
a
**
b
***
b
***
IL-2
IL-2L
control 0h
PHA 4h
PHA 24h
Time

B)

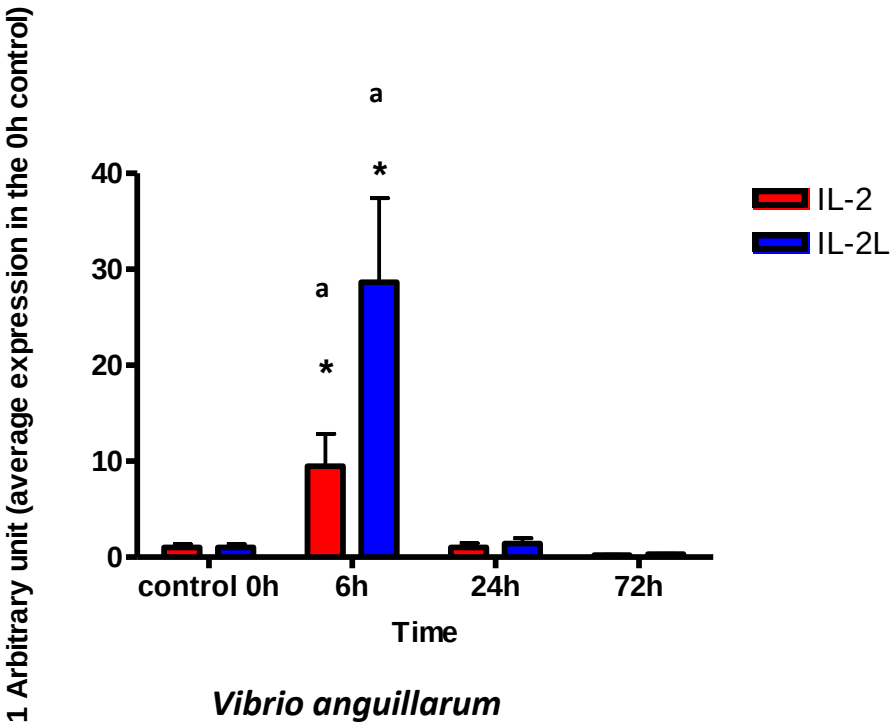
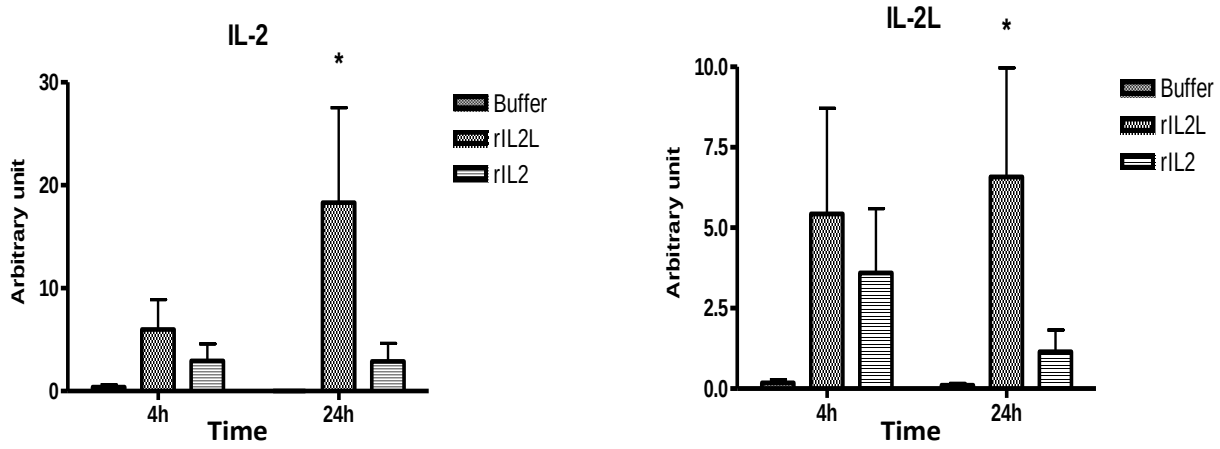

1 Arbitrary unit (average expression in the 0h control)
40
30
20
10
0
a
*
a
*
IL-2
IL-2L
control 0h
6h
24h
72h
Time
Vibrio anguillarum

Figure 6.

A) Head kidney



B) Spleen

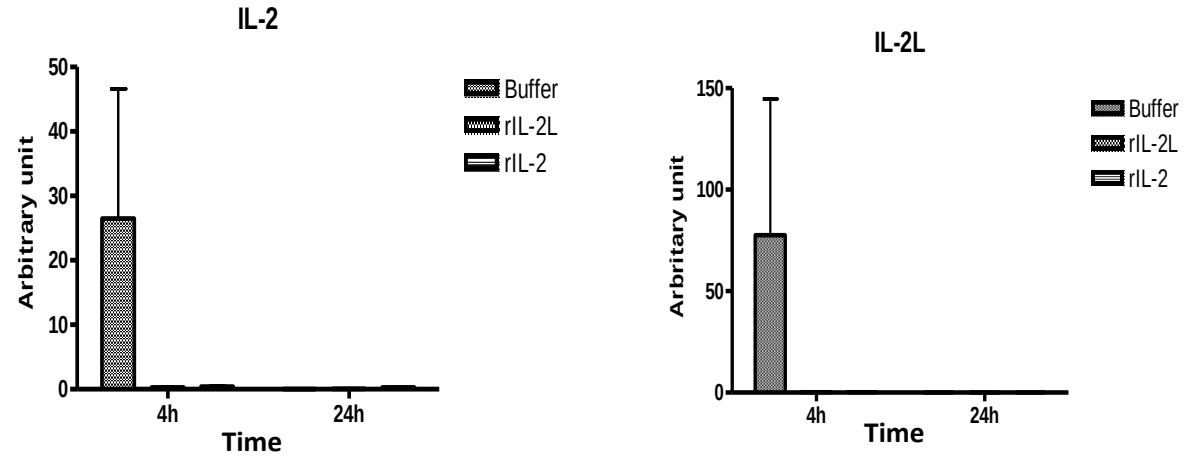
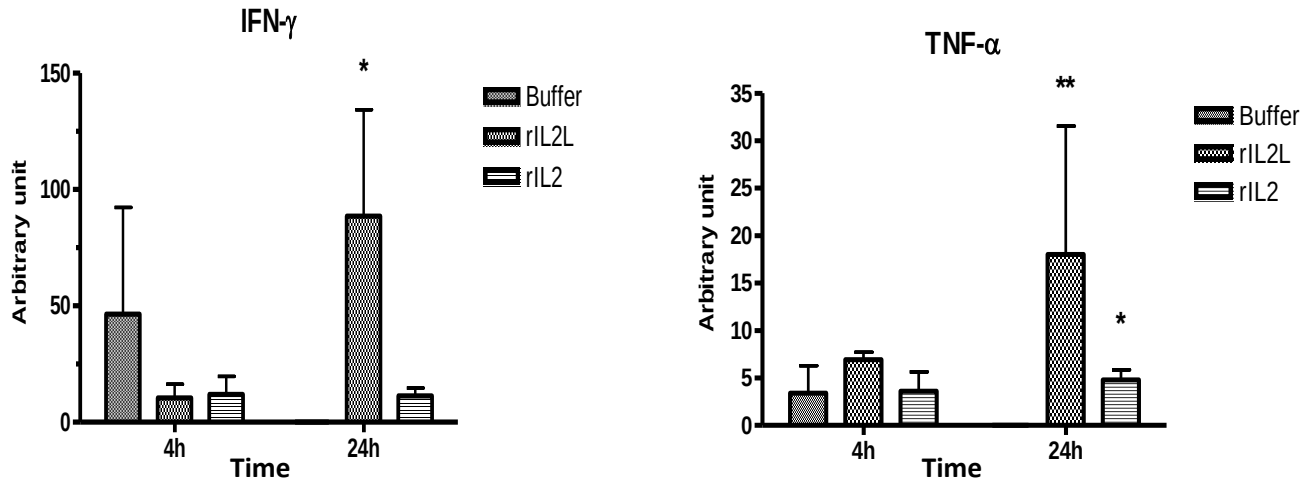


Figure 7.

A) Head kidney



B) Spleen

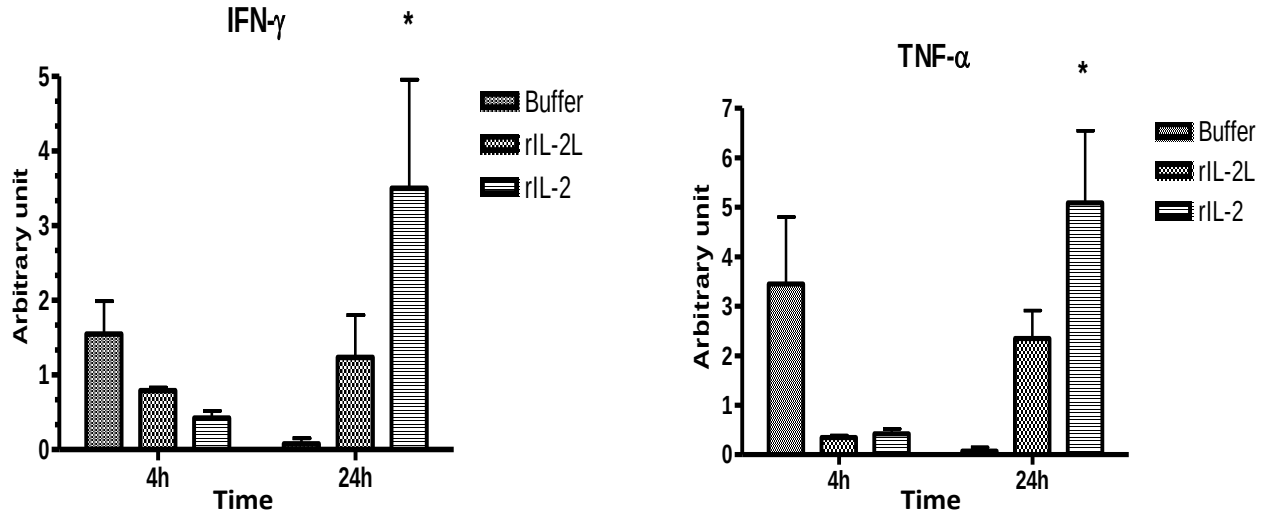
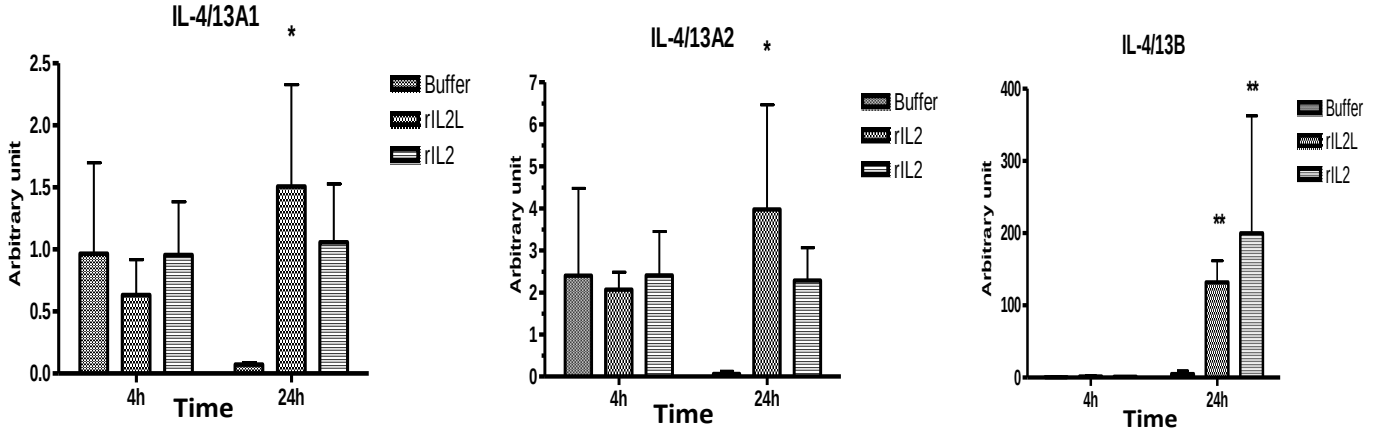


Figure 8.

A) Head kidney



B) Spleen

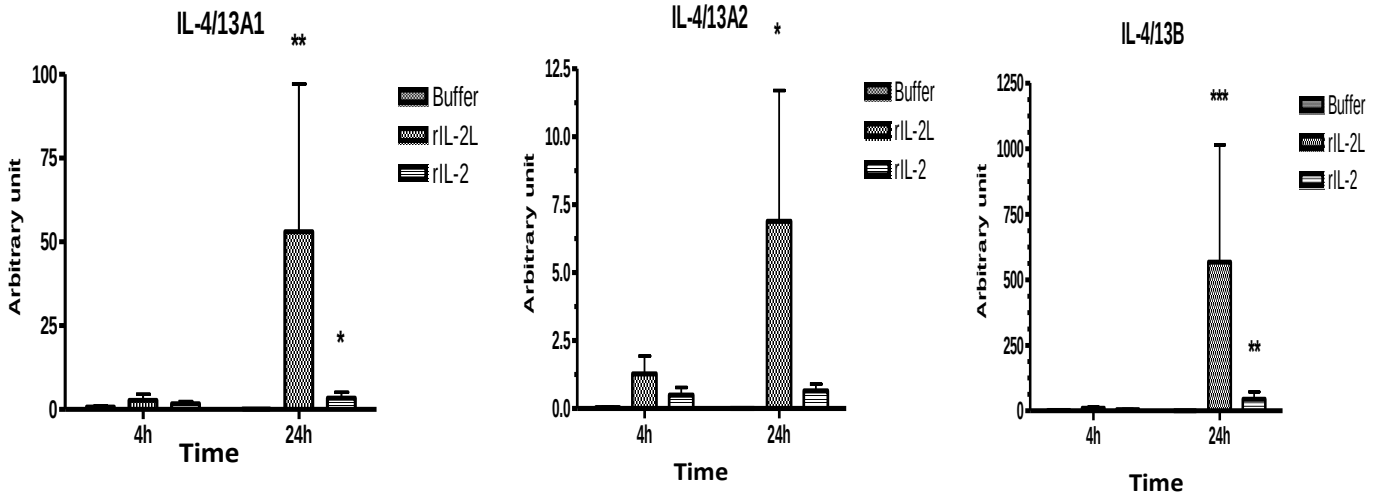
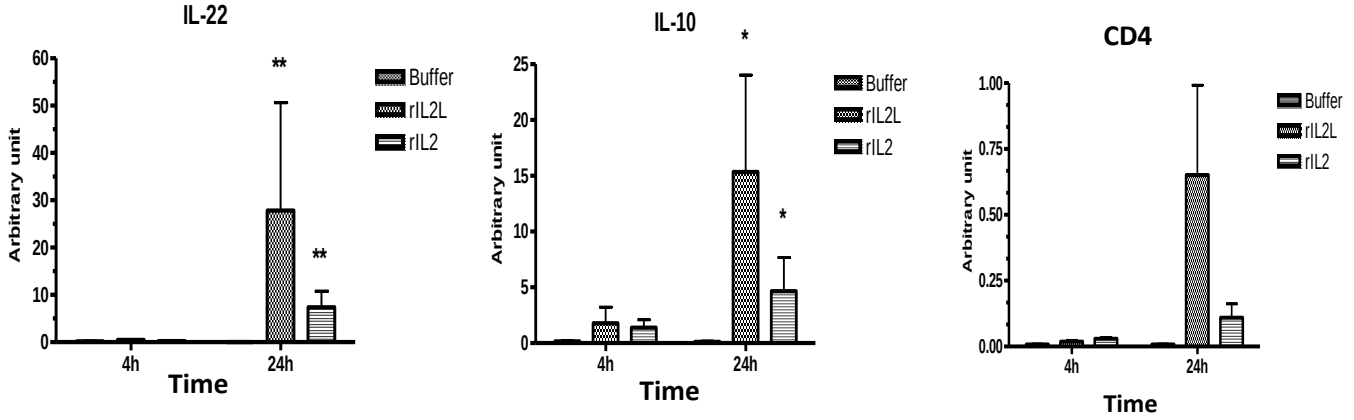


Figure 9.

A) Head kidney



B) Spleen

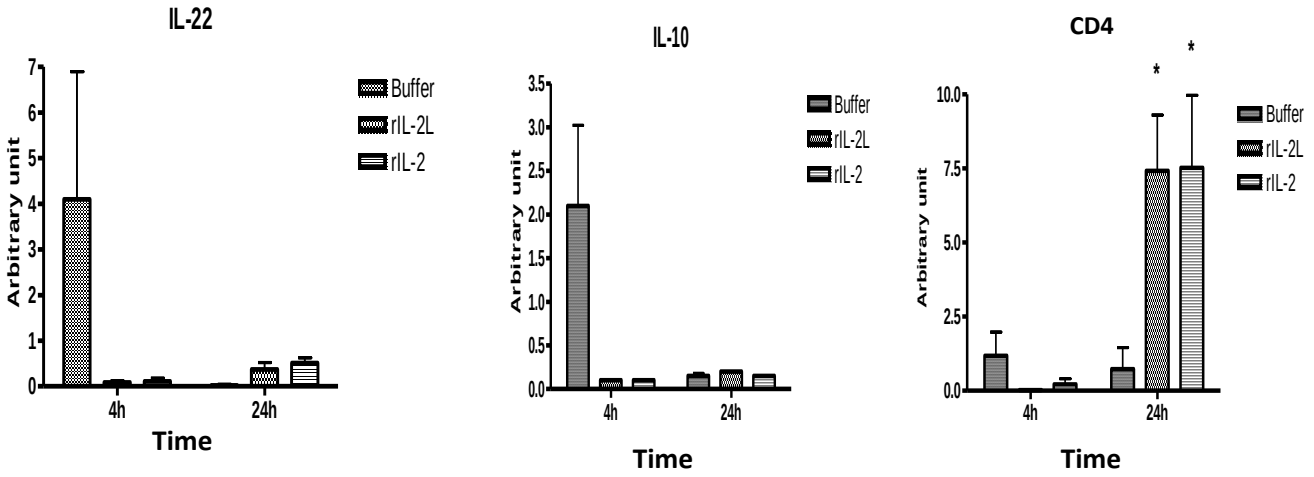
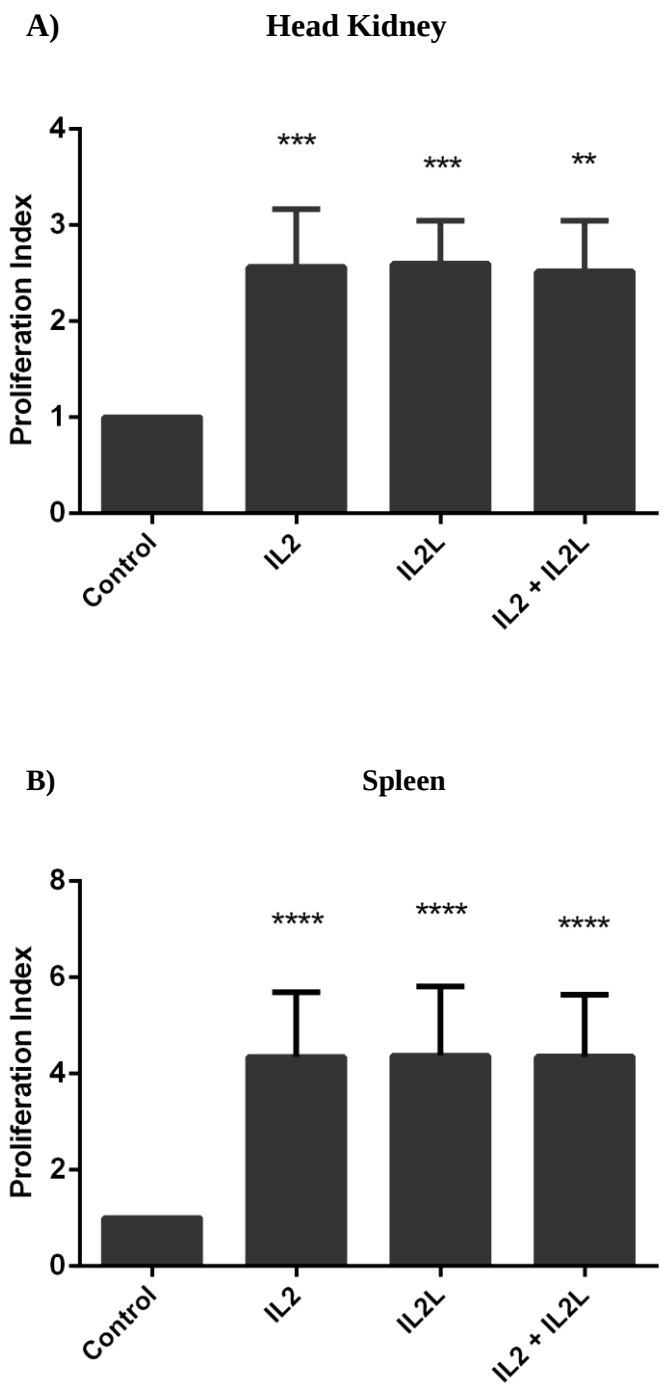


Figure 10.


A)
Head Kidney
Proliferation Index
0
1
2
3
4
***
***
**
Control
IL2
IL2L
IL2 + IL2L
B)
Spleen
Proliferation Index
0
2
4
6
8
****
****
****
Control
IL2
IL2L
IL2 + IL2L

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: