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Carp Il10a and Il10b exert identical biological activities in vitro, but are differentially regulated in vivo

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ABSTRACT

We recently reported on the functional characterization of carp Il10. We showed that carp Il10 is able to downregulate proinflammatory activities by carp phagocytes and promote B cell proliferation, differentiation and antibody production as well as proliferation of memory T cells. Taking advantage of the recent annotation of the carp genome, we completed the sequence of a second *il10* paralogue, named *il10b*, the presence of which was expected owing to the recent (8 million years ago) fourth round of whole genome duplication that occurred in common carp. In the present study we closely compared the two Il10 paralogues and show that Il10a and Il10b have almost identical gene structure, synteny, protein sequence as well as bioactivity on phagocytes. Although the two *il10* paralogues show a large overlap in tissue expression, *il10b* has a low constitutive expression and is highly upregulated upon infection, whereas *il10a* is higher expressed under basal conditions but its gene expression remains constant during viral and parasitic infections. This differential regulation is most likely due to the observed differences in their promoter regions. Altogether our results demonstrate that gene duplication in carp, although recent, led to sub-functionalization and expression divergence rather than functional redundancy of the Il10 paralogues, yet with very similar protein sequences.

1. Introduction

Interleukin 10 (IL10) is one of the most important anti-inflammatory cytokines that was first discovered in Th2 cell clones showing a potent inhibitory effect on IL2 and IFNγ synthesis in Th1 cell clones (Fiorentino et al., 1989). Since then, a plethora of studies have been conducted on this cytokine revealing incredible multifaceted activities. IL10 acts on different cell populations from both the innate and adaptive branches of the immune system redirecting a type I or inflammatory response to a type II or anti-inflammatory/regulatory response. The main biological activity of IL10 is exerted on APCs, mainly macrophages, directly preventing the production of pro-inflammatory cytokines and indirectly downregulating antigen presentation, thereby preventing Th responses (Mosser and Zhang, 2008). IL10 exerts its activities also on cells of the adaptive branch of the immune system, as it directly inhibits proliferation of $CD4^+$ T cells (Brooks et al., 2010) and cytokine synthesis by Th1 (IL2 and IFNγ) and Th2 (IL4 and IL5) cells (Del Prete et al., 1993; Groux et al., 1996). In contrast, it does not seem to directly affect Th17 cells (Naundorf et al., 2009). IL10 activity, however, does not result in suppression of immune responses only, as it is known to prevent apoptosis, increase proliferation and MHC class II expression in B cells, stimulating Ig class switching and terminal differentiation (Go et al., 1990; Moore et al., 2001; Rousset et al., 1995). IL10 also increases the cytotoxic activity of NK cells (Carson et al., 1995) and induces proliferation of certain subsets of $CD8^+$ T cells (Emmerich et al., 2012). All the aforementioned activities are well described for mammalian IL10 and some of them have been recently confirmed for birds, including chicken, duck (Rothwell et al., 2004; Wu et al., 2016; Yao et al., 2012), and teleost Il10, including cyprinid goldfish, common carp and grass carp Il10 (Grayfer et al., 2011; Piazzon et al., 2015a, 2015b; Wei et al., 2013) indicating a conservation of function of this

Abbreviations: APCs, antigen presenting cells; RTqPCR, real-time quantitative PCR; CTL, cytotoxic T lymphocyte; Socs3, suppressor of cytokine signaling 3; AP-1, activator protein-1; AP1R, MAF and AP1 related factors; CEBP, Ccaat/Enhancer Binding Protein; CREB, cAMP-responsive element binding proteins; IRF, Interferon regulatory factors; c-Maf, c-musculoaponeurotic fibrosarcoma; NF1F, Nuclear factor 1; PBXC, PBX – MEIS complexes; SP1F, GC-Box factors SP1/GC; STAT, Signal transducer and activator of transcription.

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cytokine throughout evolution. Among the characterized activities of teleost Il10 is the ability to strongly inhibit pro-inflammatory gene expression, respiratory burst and nitrogen radical production by macrophages and neutrophils, to trigger B cell proliferation and antibody production as well as to promote memory T cell proliferation. Altogether, this underlines the crucial role played by IL10 in the regulation of the immune system of mammals as well as teleost fish (Piazzon et al., 2016).

Both mammalian and fish IL10 function as homodimers composed of two non-covalently bound monomers (van Beurden et al., 2011; Windsor et al., 1993). Mammalian IL10 dimers signal via a receptor complex consisting of two copies of IL10 receptor 1 (IL10R1) and two copies of IL10R2. Activation of JAK1 (associated with IL10R1) and TYK2 (associated with IL10R2) leads to phosphorylation of STAT3 and subsequent transcription of several genes, among which the *suppressor of cytokine signaling 3* (*SOCS3*) that will ultimately downregulate pro-inflammatory cytokine gene expression. Although the exact stoichiometry of the receptor complex in teleosts is not known, recent studies in goldfish, zebrafish, common carp and grass carp confirmed that, at least in cyprinids, Il10 signals through Crfb7 (homologous to IL10R1) and Crfb4 (homologous to IL10R2), activating Stat3 signaling and ultimately leading to *socs3* upregulation (Grayfer and Belosevic, 2012; Piazzon et al., 2015a; Wei et al., 2014).

The main cellular sources of IL10 are $CD4^+$ T cells and monocytes/macrophages although IL10 can be produced by most leukocytes (Blanco et al., 2008; Chomarat et al., 1993; Fillatreau et al., 2002; Grant et al., 2008; Mehrotra et al., 1998; Rhodes et al., 2008; Speiran et al., 2009; Yanaba et al., 2009; Zhang et al., 2009). The production of IL10 is highly regulated with several aspects of *IL10* gene regulation being conserved among all IL10-producing cells but others being cell specific (Mosser and Zhang, 2008). For example, the transcription factors Sp1 and Sp3, STAT3, CEBPβ and δ, IRF-1, c-Maf, AP-1, CREB and NF-κB are known to positively regulate *IL10* transcription in most cells. Other transcription factors, such as STAT1, play a negative regulatory role on *IL10* expression in some cell types (monocytes in the case of STAT1) but induce expression in others (i.e. T cells) (Stumhofer et al., 2007; VanDeusen et al., 2006). Altogether, the variety of transcription factor binding sites present in the *IL10* promoter explains the differential regulation induced by several stimuli in different cell types.

Throughout evolution, after the two rounds of whole-genome duplications that occurred in the common ancestor of vertebrates, teleost fish underwent a third duplication event (Opazo et al., 2013) implying that several genes, among which multiple cytokines and cytokine receptors, are present in two copies in teleost fish genomes (Harun et al., 2011). In common carp, goldfish, catastomid fishes (suckers), and salmonids (i.e. rainbow trout and Atlantic salmon), a fourth round of whole-genome duplication (WGD) occurred (Allendorf and Thorgaard, 1984; David et al., 2003; Li et al., 2015; Ohno et al., 1967; Uyeno and Smith, 1972) making it very common to find multiple paralogues of many genes especially in these species. In common carp in particular, the most recent species-specific WGD of all vertebrates occurred, dated to approximately 8 million years ago, making this species a suitable model to investigate early-stage functional divergence and expression differentiation in vertebrates (Li et al., 2015). In trout, in which the fourth WGD occurred much earlier (approximately 88–103 million years ago (Macqueen and Johnston, 2014) two paralogues for *il10*, *il10a* and *il10b*, were previously described (Harun et al., 2011). The paralogues showed very similar structure, while presenting interesting differences in expression and regulation. Their biological activities however, were not investigated and directly compared. In carp, the presence of two *il10* sequences was already reported in the database. One sequence, referred to as *Il10a*, corresponds to the first full length cDNA reported by Savan et al. (Savan et al., 2003) and the second, referred to as *Il10b*, was later reported as a partial cDNA (Kongchum et al., 2011). We recently reported on the biological activity of carp Il10a and showed that, similar to mammalian IL10, it was able to downregulate pro-inflammatory activities by phagocytes, while having regulatory and stimulatory activities on B cells and memory T cells (Piazzon et al., 2015a). To date, the biological activities and structures of the two *Il10* paralogues in carp were never systematically compared.

In this study, we report the complete genomic sequences of carp *il10a* and *il10b*; we compare the gene organization and protein sequence of the two carp Il10 paralogues, as well as their bioactivity on various cell types. We also compare the promoter regions of the two molecules and study their gene expression in different tissues, cell types as well as during various infections. Taken together, our data suggest that gene duplication of *Il10* in carp did not lead to neo-functionalization or gene loss, rather to intense expression differentiation, most likely owing to clear differences in their promotor regions. This, points towards a sub-functionalization of the Il10 paralogues within the immune system of carp.

2. Materials and methods

2.1. Sequence analysis and bioinformatics

Six different complete or partial *il10* nucleotide sequences for carp *il10* have been deposited in the databases, all with slight differences, four of which (JX524550, JX524551, JF957369, HQ323755) are most similar to the originally submitted *il10* sequence by Savan et al. (AB110780), and will be referred to as *il10a*. The sixth sequence (HQ323756) was already named *il10b* as it was most different from all other sequences, but it is partial (Kongchum et al., 2011). This, and additional information from the available common carp genome Bioprojects PRJEB7241 and, in particular, PRJNA73579, was combined to verify and complete the genomic sequences of *il10a* and *il10b* of common carp. CLC bio workbench software was used to identify introns and exons. Specific primers for recombinant protein production and gene expression analysis were designed (Tables I and II) and the products were verified by sequencing. The complete cDNA sequences were submitted to the database under accession numbers: KX622693 for *Il10a* and KX622694 for *Il10b*. The nucleotide sequence was translated using the ExPASy translate tool (http://web.expasy.org/translate/) and all the alignments were performed with ClustalW v2.1 (http://www.genome.jp/tools/clustalw/). The signal peptide cleavage site was predicted using SignalP v4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011) and the secondary structure with YASPIN (http://www.ibi.vu.nl/programs/yaspinwww/) (Lin et al., 2005). The location of specific conserved residues and receptor binding sites were already described for carp Il10 (van Beurden et al., 2011) based on previously reported information on the human IL10/IL10 receptor complex (Josephson et al., 2001).

Genomic regions flanking the *IL10* gene were examined for synteny by comparing the genomes of human (*Homo sapiens*, assembly GRCh38), mouse (*Mus musculus*, assembly GRCm38), chicken (*Gallus gallus*, assembly Galgal4), tetraodon (*Tetraodon nigroviridis*, assembly TETRAODON8) and zebrafish (*Danio rerio*, assembly Zv9) from Ensemble Genome Browser (http://www.ensembl.org/index.html) and the carp genome (PRJNA73579) (Henkel et al., 2012).

For the promoter analysis, 900 bp upstream of the start codon of each paralogue were analyzed with MatInspector (genomatix http://www.genomatix.de/) focusing on the vertebrate databases and matrix families present in the human and murine *IL10* promoters (Mosser and Zhang, 2008; Saraiva and O'Garra, 2010): AP1R,

Table 1
Primers used for cloning.

Primer	Sequence (5′-3′)	Use
TGFLeader_cIl10_FW	CAATGCCTGTTGGGATTTGTGCACTATAGCGGAGCAAGAAGAGTCGACTGCA	Amplification of *il10a* and *il10b* sequences without signal peptide, and introduction of the 3′ end of the carp Tgf signal peptide.
XhoI_cIl10a_RV	TGGCCCTCGAGTTAGTGCTTGCCTCTCTT	Amplification of *il10a* and introduction of XhoI site in 3′ end.
cIl10b_FW	GGTCATG + CTTCTGCTT + ACT	Amplification of *il10b* still retaining part of own signal peptide.
XhoI_cIl10b_RV	TGGCCCTCGAGCTAT + AAGTGCTTG + ACTC	Amplification of *il10b* and introduction of XhoI site in 3′ end.
NheI_TGFLeader FW	TTGCGGCTAGCATGAGGGTGGAGAGTTTATTACTGGCATTGCAATGCCTGTTGGGATTT	Complete introduction of Tgf signal peptide and addition of NheI site in 5′ end.

+ indicates LNA (locked nucleic acid) substitutions.

Table 2
Primers used for real time quantitative PCR.

Primer	Sequence	Amplification efficiencies*	GeneBank Accession no.**
*40s*_FW	CCGTGGGTGACATCGTTACA	1.71	AB012087
*40s*_RV	TCAGGACATTGAACCTCACTGTCT		
*socs3a*_FW	CCTTCAGACGGACTCCAA	1.74	cypCar_00035971 & cypCar_00014365
*socs3a*_RV	CAAGGAAGGGGTCTCAAC		
*socs3b*_FW	CCGCTGGAGAAGGTGGAA	1.75	cypCar_00028701 & cypCar_00007125
*socs3b*_RV	CTGGAGGAACTCTTGGAGTG		
*il10a*_FW	CGCCAGCATAAAGAACTCA	1.78	HQ323755/JX524551 (cypCar_00007086)
*il10a*_RV	TGCCAAATACTGCTCAATGT		
*il10b*_FW	CGCCAGCATAAAGAACTCGT	1.76	HQ323756 (cypCar_00012555)
*il10b*_RV	TGCCAAATACTGCTCGATGT		

*Amplification efficiencies are calculated for each sample according to the Comparative Quantitation method included in the RotorGene6000 software. With this method, optimal efficiencies are between 1.7 and 1.85. **_cypCar_ numbers identify ORFs in the draft carp genome (bioproject PRJNA73579) that were also confirmed by RNA sequencing. Both *socs3a* and *socs3b* paralogues have two copies in carp. To simplify, the primers used in this study were designed to amplify both copies of each paralogue.

CEBP, CREB, IRF, NF1F, PBXC, SP1F and STAT. To enhance the performance of the analysis, core similarities and matrix similarities were set to a minimum of 0.8.

2.2. Production of Il10a and Il10b in eukaryotic cells

The *il10a* and *il10b* sequences, without the signal peptides (aa 23-180 for Il10a and 23-181 for Il10b), were amplified from naïve carp head kidney (*il10a*) and *Trypanoplasma borreli*-infected head kidney (*il10b*) cDNA using specific primers (Table 1). Additional primers were designed to introduce the required restriction sites for subsequent cloning into pcDNA3 (Invitrogen) together with the carp Tgf leader sequence (MRVESLLLALQCLLGFVHYSGA, accession number Q9PTQ2) to enhance protein secretion. Upon transformation into JM109 *Escherichia coli* cells, positive clones were verified by standard sequencing. Plasmids were isolated by Mini-Prep (Qiagen) and quantified using a Nanodrop 1000 spectrometer (Thermo Scientific).

For transfection, 500.000 HEK293 cells were seeded in 6 well plates in 2 ml of Advanced DMEM/F-12 supplemented with 2% FBS (Gibco, Life Technologies) and incubated overnight at 37 °C. Transfection was performed with JetPRIME™ (Polyplus transfection) using 2 μg of each plasmid (pcDNA3-Il10a, pcDNA3-Il10b and empty pcDNA3) and a plasmid:JetPrime ratio of 1:2. After 4 h, the medium was replaced for 2 ml of fresh Advanced DMEM/F-12 supplemented with 2% FBS and plates were incubated for 4 days at 37 °C. Supernatants were collected, cleared through a 0.22 μm filter, aliquoted and stored at −20 °C until use. A large batch of supernatants was produced and used throughout the study for quantification and assessment of biological activity as described below.

2.3. Anti-carp Il10 antibodies

Polyclonal rabbit serum anti-carp Il10 was produced by immunization of rabbits with purified bacterial recombinant carp Il10a (cIl10a) (Piazzon et al., 2015a) according to a 3-month standard protocol (Eurogentec). Total rabbit IgG was purified using a Melon Gel IgG Purification Resin (Thermo Scientific) and affinity purified using a Sepharose-cIl10a column. Purified antibodies were dialyzed against PBS and the concentration determined using a Nanodrop 1000 spectrometer. Aliquots of 100 μg/ml were stored at −20 °C until use.

Polyclonal chicken IgY anti-carp Il10a were produced in house by immunization of chicken with purified bacterial recombinant cIl10a. Eggs containing highest titers of anti-carp Il10 IgY were selected by dot blot analysis and pooled. Total chicken IgY was purified using polyethyleneglycol-6000 (Polson et al., 1980).

Although rabbit and chicken polyclonal antibodies were raised against Il10a as immunizing antigen, they recognized both Il10 isoforms in western blot analysis (section 2.4) and will be further referred to as anti-carp Il10 antibodies. They were used to quantify the concentration of Il10a and Il10b in cell culture supernatants by ELISA, as described below.

2.4. Detection and quantification of Il10 in cell culture supernatants

The presence of Il10 in cell culture supernatants 4 days after transfection was confirmed by western blot analysis and quantified using a sandwich ELISA. For Western blot analysis, 20 μl of cell culture supernatants from cells transfected with either empty pcDNA3, pcDNA3-Il10a or pcDNA3-Il10b were resolved on a 12% SDS-PAGE gel and transferred at 15 V for 30 min to nitrocellulose membranes (Protran; Schleicher & Schuell BioScience) in a Trans-Blot SD Transfer Cell (Bio-Rad). The membranes were blocked for 2 h at room temperature with 5% (w/v) non-fat dry milk in TBST (50 mM Tris, 0.15 M NaCl [pH 7.4], 0.2% [v/v] Tween-20) and incubated overnight at 4 °C with a 1:1000 dilution of affinity purified rabbit anti-carp Il10 or 1:200 dilution of chicken IgY anti-carp Il10 in 1% non-fat dry milk TBST. HRP-conjugated goat anti-rabbit

(1:1000; Dako) or polyclonal rabbit anti-chicken IgY-HRP (1:2000; Jackson ImmunoResearch) in 1% non-fat dry milk TBST were used as secondary antibodies (1 h at RT), and the proteins were visualized by chemiluminescence detection (Western Bright ECL Western blotting Detection Kit; Advansta) on x-ray films.

For Il10 quantification in cell culture supernatant, ELISA plates were coated with 4 μg/ml of rabbit anti-carp Il10 IgG in carbonate/bicarbonate buffer 0.1 M pH 9.6, incubated overnight at 4 °C and blocked for 1 h with 1% BSA (w/v) in 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 (TBS)-tween-20 (0.05% v/v, TBS-T) at 37 °C. The following steps were all performed at 37 °C. After washing, 100 μl of serial dilutions of recombinant bacterial cIl10a (100–1.6 ng) in Advanced DMEM/F-12 supplemented with 2% FBS were added in triplicate and 50 μl of each supernatant (containing Il10a, Il10b and control) were mixed with 50 μl of the same medium and added in quadruplicate wells. The plate was incubated for 1 h, washed and incubated for 1 h with a 1:100 dilution of anti-carp Il10 IgY in TBS-T. A polyclonal rabbit anti-chicken IgY-HRP (Jackson ImmunoResearch, 1:2000) was added and plates were incubated for 1 h before developing with ABTS substrate (Roche). The OD_{405} was measured in a FilterMax™ F5 Multi-Mode Microplate Reader. The standard curve presented a linear phase from 1.6 to 25 ng of prokaryotic recombinant protein reaching a plateau in concentrations above 25 ng. When recombinant supernatants were tested for the presence of Il10, only values within the linear range of the standard curve were used to calculate concentrations. The concentrations measured in 50 μl of culture supernatants from the batch used in this study were 362.3 and 339.6 ng/ml of Il10a and Il10b respectively. Therefore, Il10a was diluted to the concentration of Il10b and equal volumes were used for the assays.

2.5. Fish and infections

European common carp (*Cyprinus carpio carpio* L.) were bred and raised in the Aquatic Research Facility (ARC) of Carus at Wageningen University, The Netherlands, at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 carp, which are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain), were used (Irnazarow, 1995). All experiments were performed with the approval of the animal experiments committee of Wageningen University.

For SVCV infection, 30–40 g carp were exposed, by immersion, to SVCV-infected tissue culture (10^3 $TCID_{50}$/ml) as described previously (Forlenza et al., 2008a). Head kidney samples were collected at 0, 6, 24, 48 h, 4, 7 days, 2 and 7 weeks post-infection for RNA extraction and cDNA synthesis. For *Trypanoplasma borreli* and *Trypanosoma carassii* infections, 140–180 g carp were i.p. injected with 10^4 parasites/fish and PBS-injected fish served as a non-infected control group (Forlenza et al., 2008b; Joerink et al., 2006a). Head kidney samples were collected at different time points post-infection with *T. borreli* or with *T. carassii*, for RNA extraction and cDNA synthesis.

2.6. Cell isolation and culture

For organ isolation, fish were killed with an overdose of Tricaine Methane Sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA). Peripheral blood leukocytes (PBL), mid kidney leukocytes (MKL) and head kidney leukocytes (HKL) were isolated as described previously (Forlenza et al., 2008c; Koumans-Van Diepen et al., 1994). Head kidney-derived macrophages (referred to as macrophages, (Joerink et al., 2006b) and mid kidney magnetic activated cell sorted (MACS) neutrophils (Forlenza et al., 2008b; Nakayasu et al., 1998) were obtained as previously described. MACS sorting was also used to isolate B cells and thrombocytes from PBLs using anti-carp Igm (WCI12, (Secombes et al., 1983)) and anti-carp thrombocytes (WCL6, (Rombout et al., 1996)) antibodies. The purity of the sorted cells was verified to be >98% by flow cytometry using a BD FACS Canto A™ (BD Biosciences). After isolation, cells were washed in RPMI 1640 with L-glutamine and 25 mM Hepes (Lonza, Nalgene) medium adjusted to 280 mOsmol/Kg and supplemented with 2 mM L-glutamine, 100 U/ml penicillin G and 50 mg/ml streptomycin sulphate (RPMI with Hepes), counted and resuspended in the same medium without Hepes (RPMI) unless stated otherwise.

2.7. Nitrogen radical production

HKL (1×10^6/well) were seeded in 96 well plates (Corning®) and stimulated with HEK supernatants containing 120 ng/ml of Il10a or Il10b alone or in combination with 50 μg/ml LPS. The concentration of Il10 was based on a pilot experiment testing a range of volumes of Il10a- and Il10b-containing cell culture supernatants, after which Il10 concentration was determined by ELISA and adjusted to 120 ng/ml using cell culture supernatants. As a control, supernatant from HEK cells transfected with the empty plasmid in a volume equivalent to the volume of Il10-containing supernatants was used. Cells were incubated in a final volume of 150 μl RPMI supplemented with 1.5% pooled carp serum for 4 days at 27 °C in the presence of 5% CO_2. The production of nitrogen radicals was determined using the Griess reagent as previously described (Saeij et al., 2003). The OD_{540} was measured after 10 min in a FilterMax™ F5 Multi-Mode Microplate Reader.

2.8. Oxygen radical production (Luminol)

To measure the production of oxygen radicals by carp HKL a real time luminol-enhanced chemiluminescence assay was performed as previously described (Pietretti et al., 2013). Briefly, 50 μl of luminol (10 mM, Sigma-Aldrich, in 0.2 M borate buffer pH 9.0) and 50 μl of stimulus (0.1 μg/ml PMA in combination with 120 ng/ml Il10a, Il10b, or equivalent volume of control supernatant) were added to the wells of a white 96-well plate with opaque bottom (Corning®). Finally, 1×10^6 HKL in 50 μl of RPMI were added and chemiluminescence emission was measured with a FilterMax™ F5 Multi-Mode Microplate Reader every 3 min during 90 min at 27 °C.

2.9. Gene expression analysis by real time-quantitative PCR

To measure *socs3* induction by Il10, 3×10^6 HKL were seeded in 48 well plates (Corning®) and incubated with HEK supernatants containing 120 ng/ml of Il10a or Il10b or control supernatants in an equivalent volume, in a final volume of 300 μl RPMI for 3 h at 27 °C in the presence of CO_2. Total RNA was isolated using the RNeasy Kit (Qiagen), including on-column DNase treatment, and stored at −80 °C. Prior to cDNA synthesis, 500 ng of total RNA were treated with DNase I, Amplification Grade (Invitrogen) and cDNA synthesized using 300 ng of random primers and Superscript III First Strand Synthesis for RT-PCR (Invitrogen). cDNA samples were further diluted 25 times in nuclease-free water prior to real time-quantitative PCR (RT-qPCR) analysis.

RT-qPCR analysis was performed with a Rotor-Gene 6000 (Corbett Research) using ABsolute qPCR SYBR Green Mix (Thermo Scientific). The primers used are shown in Table 2. Fluorescent data from RT-qPCR experiments were analyzed using Rotor-Gene Analysis software v1.7. The take-off value for each sample and the average reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from Rotor Gene Software (Forlenza et al., 2012). The relative expression ratio (R) of a target

gene was calculated based on the average *E* and the take-off deviation of sample versus control, and expressed relative to the *s11* protein of the *40s* subunit as a reference gene.

2.10. Statistical analysis

Tests were performed on means, following verification of normality using the Shapiro-Wilk test for small samples, and evaluated using Student's T test. If not, Mann-Whitney-Wilcoxon non-parametric tests were used (R3.0.2, (R Core Team, 2014). Where applicable, data were analyzed as paired data to eliminate the interference caused by variability among individual fish. For gene expression analysis, relative expression ratios (R) were calculated as described, and transformed (LN(R)) values were used for statistical analysis. For multiple comparisons, one-way ANOVA followed by the Tukey's test was used. In all cases, differences were considered significant at $P < 0.05$.

3. Results

3.1. Carp Il10a and Il10b show very similar genomic structures and protein sequences

After the recent fourth round of WGD, most ancestral genes have been retained in duplicate in carp, with evidence of slow gene loss but rapid and intense expression and functional differentiation (Li et al., 2015). We previously described the biological activity of *il10a* (Piazzon et al., 2015a) and here, aimed to confirm the presence, characterize the sequence and compare the biological activity of a second paralogue of carp *il10*. Genome analysis confirmed the presence of carp *il10* genes located in two different contigs (Contig30115 for *il10a* and Contig21661 for *il10b*), with *il10a* denoted as cypCar_00007086 and *il10b* as cypCar_00012555 (bioproject PRJNA73579). Both paralogues are composed of 5 exons with a very similar genomic organization and encode for a cDNA of 540 (*il10a*) and 543 (*il10b*) bp with 92% nucleotide identity (Fig. 1 A and B). The 3' end of both molecules contains three instability motives (ATTTA) before the polyadenylation signal (AATAAA) (not shown). Carp Il10a and Il10b proteins are 180 and 181 amino acids long, respectively, and show 88% amino acid identity and 94% amino acid similarity. Il10a and Il10b have highly conserved features including the predicted formation of 6 helices, a 22 amino acid long signal peptide, conserved hydrophobic residues, ion pairs and the four conserved cysteine residues that form the two disulphide bridges common in the mammalian IL10, as well as the two extra cysteine residues described for teleost Il10. The residues predicted to make contact with the Il10 receptor also show a high degree of conservation (Fig. 1 C). The high protein sequence similarity probably explains the recognition of both isoforms by the rabbit and chicken antibodies raised against Il10a (Piazzon et al., 2015a), and suggests a potential conservation of bioactivity.

3.2. Il10a and Il10b exert the same bioactivity

Previously, we showed that Il10a significantly downregulates oxygen and nitrogen radical production in carp neutrophils and macrophages, and signals via phosphorylation of Stat3 leading to upregulation of *socs3a* and *socs3b* (Piazzon et al., 2015a). Given the high protein sequence similarity between Il10a and Il10b, also supported by the cross-reactivity of both anti-Il10 antibodies against both molecules (Fig. 2A), we expected similar prototypical activities for Il10b. In order to test this, HKL were stimulated with 120 ng/ml of Il10a, Il10b, equivalent volume of control supernatant alone, or in combination with PMA or LPS as co-stimulants. Without additional co-stimulation, none of the supernatants had an effect on nitric oxide production or respiratory burst activity. However, both Il10 paralogues significantly reduced LPS-induced nitric oxide production (Fig. 2B) and PMA-induced respiratory burst (Fig. 2C), while significantly increasing the expression of *socs3a* and *socs3b* (Fig. 2D and E). These results demonstrate that both Il10a and Il10b show the same prototypical bioactivities on carp leukocytes.

3.3. While synteny is conserved, the promoter regions of the two paralogues are different

The location of the *IL10* gene with respect to that of known *IL10*-neighbouring genes was compared among human, mouse, chicken, tetraodon, zebrafish and carp to investigate whether both carp *il10* paralogues would show synteny. The genes immediately upstream (*MAPKAPK2* and *DYRK3*) and downstream (*IL19*, *IL20* and *IL24*) of human *IL10* are common to all species studied and thus highly conserved (Fig. 3A). *IL19*, *IL20* and *IL24* are believed to have derived from two consecutive duplication events in the mammalian lineage only, explaining why in other vertebrates a single copy of this gene is found (for example, Il19 in carp and zebrafish, Il20 in tetraodon, and IL24 in chicken.

As opposed to the strong conservation of synteny, the promoter regions of *il10a* and *il10b* show only 49.8% nucleotide similarity, revealing an interesting difference between the two molecules. Nine hundred bp upstream of the start codon were analyzed for the presence of predicted binding sites for different transcription factors known to be present in the mammalian *IL10* promoter (Saraiva and O'Garra, 2010). The length of the promotor sequence analyzed was based on known human and murine *IL10* promoters (Mosser and Zhang, 2008). Of interest, a number of clear differences were noted between the promotor regions of *il10a* and *il10b*. Although both have several STAT1 binding sites (Fig. 3B), only the *il10b* promotor contains one putative STAT5 and one STAT6 binding site. Furthermore, the *il10b* promotor also contains several PBX binding sites that are completely absent from the *il10a* promoter. Conversely, the *il10a* promotor sequence has SP1, NF1 and ISGF3 binding sites that are not present in the *il10b* promotor. These differences can lead to a differential regulation of the gene expression of these two paralogues, pointing to potentially different roles in the immune response.

3.4. il10 paralogues show differential expression in immune tissues and cells

Although *il10a* and *il10b* share the same bioactivity, promotor sequence differences point to a differential regulation of the two paralogues. Expression analysis in different immune tissues (Fig. 4 A) showed that *il10a* is generally higher expressed than *il10b* under basal conditions, being especially high in mid kidney and head kidney, followed by gut and gills. The basal expression of *il10b* is particularly low in spleen and liver. In sorted B cells, thrombocytes and macrophages (Fig. 4 B) the basal expression of both paralogues is similar, being lowest in B cells. In sorted neutrophils however, the expression of *il10a* is surprisingly high, perhaps explaining, at least in part, the especially high *il10a* expression in head kidney and mid kidney, which are tissues rich in neutrophils. Furthermore, since the differential expression of the two *il10* paralogues was more evident in the tissues than in the selected sorted immune cell types, it cannot be excluded that in tissues, other (non-)immune cells can contribute especially to *il10a* expression. Altogether, basal gene expression confirmed the differential regulation of the two *il10* paralogues, which was suspected based on the clear differences between the two promotor regions.

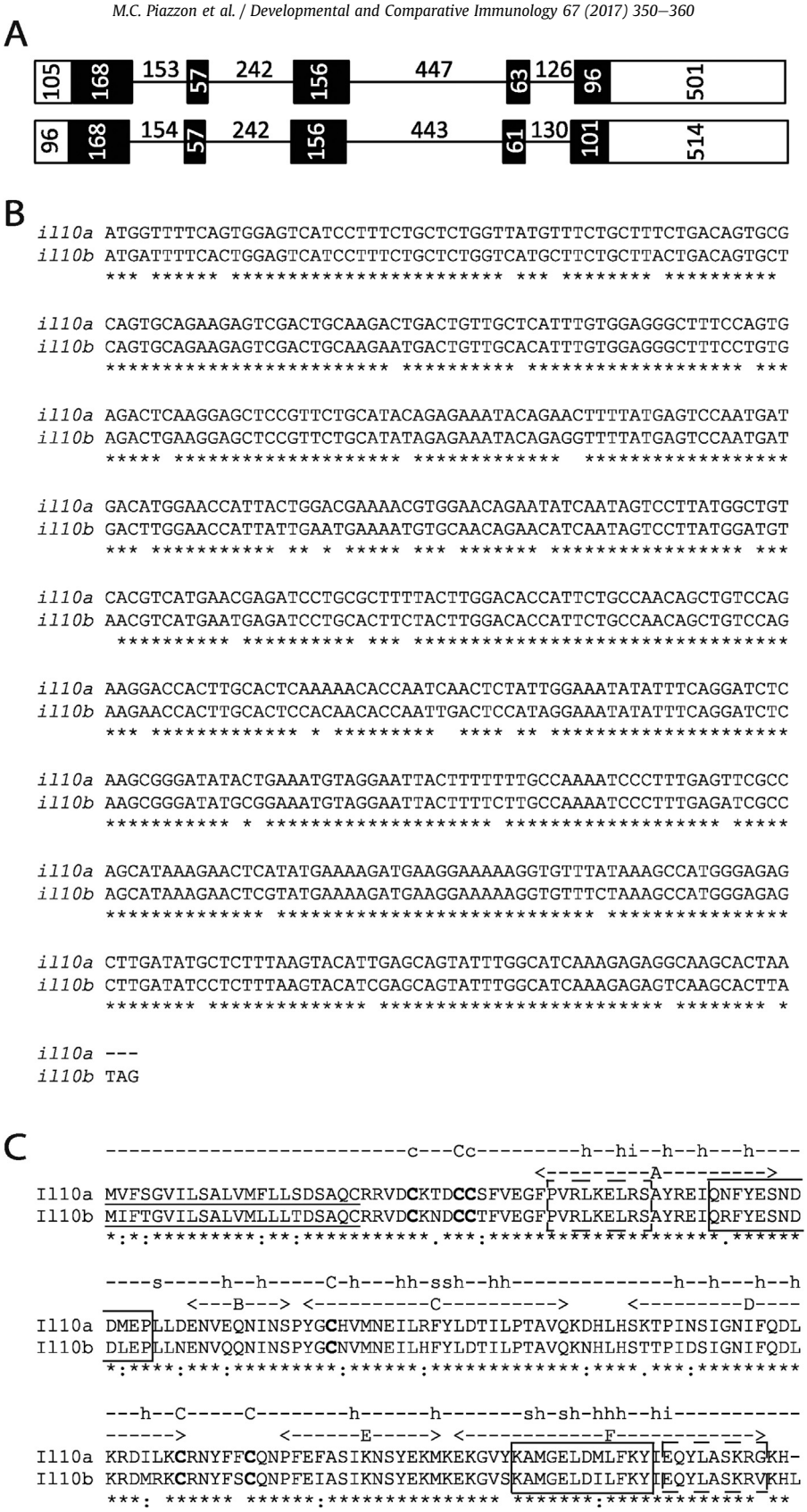

Fig. 1. Analysis of carp Il10a and Il10b genes and proteins: A) Schematic representation of *il10a* and *il10b* genes in carp. Boxes represent exons, white boxes 5′ and 3′ UTRs, black boxes coding regions and lines represent introns. The numbers and sizes indicate the length in bp. B) Sequence alignment of carp *il10a* and *il10b* CDS where asterisks (*) indicate identities (92%). C) Sequence alignment of carp Il10a and Il10b proteins. Asterisks (*) indicate identities (88%), double dots (:) indicate conserved substitutions and single dots (.) semi-conserved substitutions giving a percentage of similarity of 94%. Underlined letters represent the signal peptides. The position of the helices A to F indicated by dashes above the sequence was determined with YASPIN. Boxes indicate the human IL10R1 binding sites as described in (Josephson et al., 2001) and (van Beurden et al., 2011). The four conserved cysteine residues that form the disulphide bridges also in mammalian IL10 are indicated by (C) and the extra two cysteine residues conserved in fish are indicated by (c). (s) indicates conserved structural residues, (h) conserved hydrophobic residues and (i) the ion pair.

3.5. *il10a is higher expressed in basal conditions whereas il10b is the paralogue regulated upon infection*

In order to study differential regulation of carp *il10a and il10b,* we investigated their expression during a viral (SVCV) or parasitic (*T. carassii* and *T. borreli*) infection. In head kidney from carp infected with SVCV, while the expression of *il10a* remained constant, a peak of *il10b* expression was observed at 4 days post-infection when the virus titer was highest (Fig. 5A). *il10b* expression returned to basal levels by day 7 post-infection onwards. Upon infection with *T. borreli* (Fig. 5B) *il10a* expression remained unchanged whereas *il10b* was highly upregulated at 4 weeks post-infection, one week after the peak of parasitemia, and slowly returned to basal levels in the following weeks. The picture was quite different upon *T. carassii* infection (Fig. 5C) where *il10b* expression was significantly upregulated first at 2 weeks after infection, coinciding with the establishment of parasitemia, and again at 5 weeks post-infection, one week after the peak of parasitemia. Although it may seem that *il10a* expression shows certain fluctuation, no significant differences were found, suggesting that, although *il10a* is highly expressed under basal conditions, the paralogue that responds to the infection and might play an immunoregulatory role during in vivo challenges is *il10b*.

4. Discussion

In the present study, we compared the gene structure, synteny, biological activity and regulation of two paralogues of carp Il10, Il10a and Il10b. We show that the protein sequences of the two paralogues are extremely similar and that they share the same biological activity on carp leukocytes. Nevertheless, *il10a* and *il10b* show important differences in their promoter regions. In agreement, the regulation of the gene expression of the two paralogues is different: *il10a* is highest expressed under basal conditions whereas *il10b* appears to be the paralogue regulated upon infection.

Carp Il10 was first reported by Savan (Savan et al., 2003) who already described a conserved structure of carp *il10*. Taking advantage of the recent annotation of the carp genome, we confirmed the presence of a second *il10* paralogue, named *il10b*, the presence of which was expected owing to the fourth round of whole genome duplication (WGD) that occurred in common carp and to a previous study reporting on a second partial Il10 gene (Kongchum et al., 2011). Given the relatively short scaffolds of the carp genome assembly it is not yet possible to confirm that *Il10a* and *Il10b* are on chromosomes pairs originating from the carp-specific WGD.

Both carp *il10* genes contain five exons separated by four introns, retaining the gene structure found in human and mice *IL10* (Mosser and Zhang, 2008) as well as other fish species (Pinto et al., 2007; Zou et al., 2003). This intron/exon genomic organization is common to other cytokines of the same family such as IL19, IL20, IL22, IL24, IL26, IL28 and IL29 (Commins et al., 2008) which bind to receptors with similar structures and, in some cases, share receptor subunits (Donnelly et al., 2004). Despite most IL10 family members activate the JAK/STAT signaling pathway through engagement of common IL10R subunits, this can often lead to different biological activities. While the size of the exons is well conserved, the size of the introns is much more compacted in fish compared to mammals (Zou et al., 2003). The 3' UTR region of both carp *il10a* and *il10b* has three instability domains before the polyadenylation site, suggesting similar post-transcriptional regulation. The number of these domains varies among species, with 5 domains in the human *IL10* gene, 3 in *Catla catla* (Swain et al., 2012), 7 in trout *il10b* and none in trout *il10a* (Harun et al., 2011). At protein level, carp Il10a and Il10b are very similar, and retain the prototypical structure

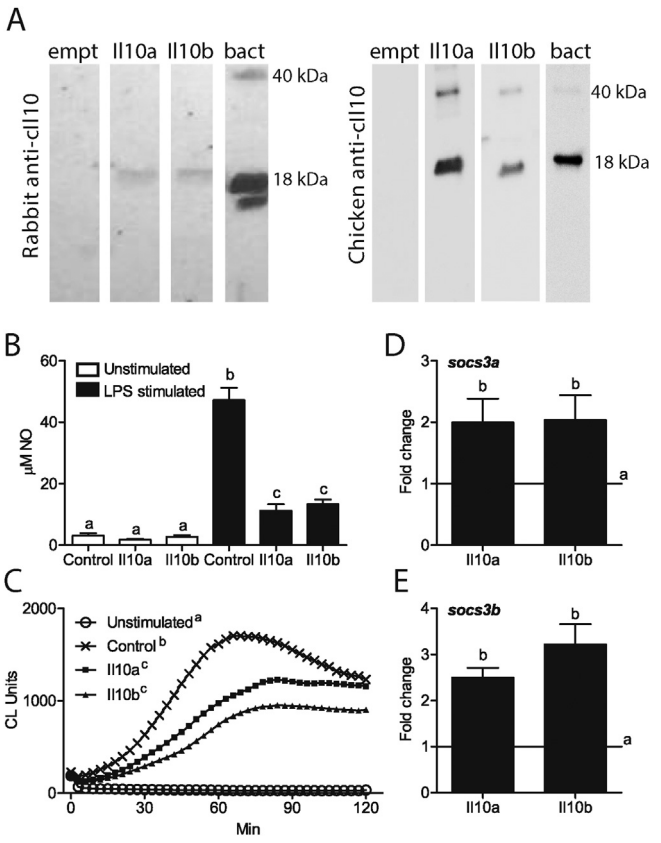

Fig. 2. Il10a and Il10b exert similar biological activities. A) Western blot analysis of cell culture supernatants (20 μl) from cells transfected with either empty pcDNA3 (empt), or with pcDNA3-Il10a or pcDNA3-Il10b, detected using rabbit (left) or chicken (right) anti-Il10 antibodies. Bacterial recombinant Il10a (bact, 1 μg) was used as positive control. The 20 kDa band indicates the Il10 monomer and the 40 kDa the Il10 dimer. The dimer is more easily visible in the right blot, most likely due to a longer exposure time of the film. B) Nitric oxide assay performed on HKL stimulated with recombinant Il10a and Il10b (120 ng/ml) either or not in combination with LPS (50 μg/ml). The OD_{540} was measured and the concentration was estimated using a nitrite standard curve. Bars indicate mean + SD of triplicate measurements from one representative experiment out of 3 performed independently. C) Respiratory burst analysis on HKL stimulated with PMA (0.1 μg/ml) alone or in combination with 120 ng/ml of Il10a or Il10b. Chemiluminescence (CL) units were measured every 3 min for 90 min. The mean of triplicate measurements from one representative experiment out of 3 is shown. D) and E) gene expression analysis of *socs3a* and *socs3b* respectively, of HKL stimulated for 3 h with 120 ng/ml of Il10a or Il10b. Gene expression was normalized relative to the *s11* protein of the *40s* subunit as a reference gene and is shown relative to the control supernatant (line at y = 1). Data are shown as mean + SD of $n = 3$ fish. In all cases, different letters indicate significant differences between groups. For all bioactivity experiments the control samples contained supernatant from empty plasmid transfected cells in a volume equivalent to the highest volume of Il10-containing supernatants used.

known for Il10 (Harun et al., 2011; Swain et al., 2012; Windsor et al., 1993; Zhang et al., 2005; Zou et al., 2003). The residues predicted to contact the IL10 receptor (Josephson et al., 2001) are highly conserved among carp, human and other species (van Beurden et al., 2011) and showed almost no differences between the two carp paralogues. In summary, the shared structural features all point towards a similar bioactivity of the two paralogues. One of the most potent effects of carp Il10a is the deactivation of phagocytes by rapidly inhibiting the production of toxic radicals and the synthesis of pro-inflammatory cytokines via early activation of the transcription factor *socs3* (Piazzon et al., 2015a). Indeed, in the present study we show that also Il10b exerts the same prototypical inhibitory activities on carp phagocytes.

Studies on the mouse and human *IL10* promoter show that the

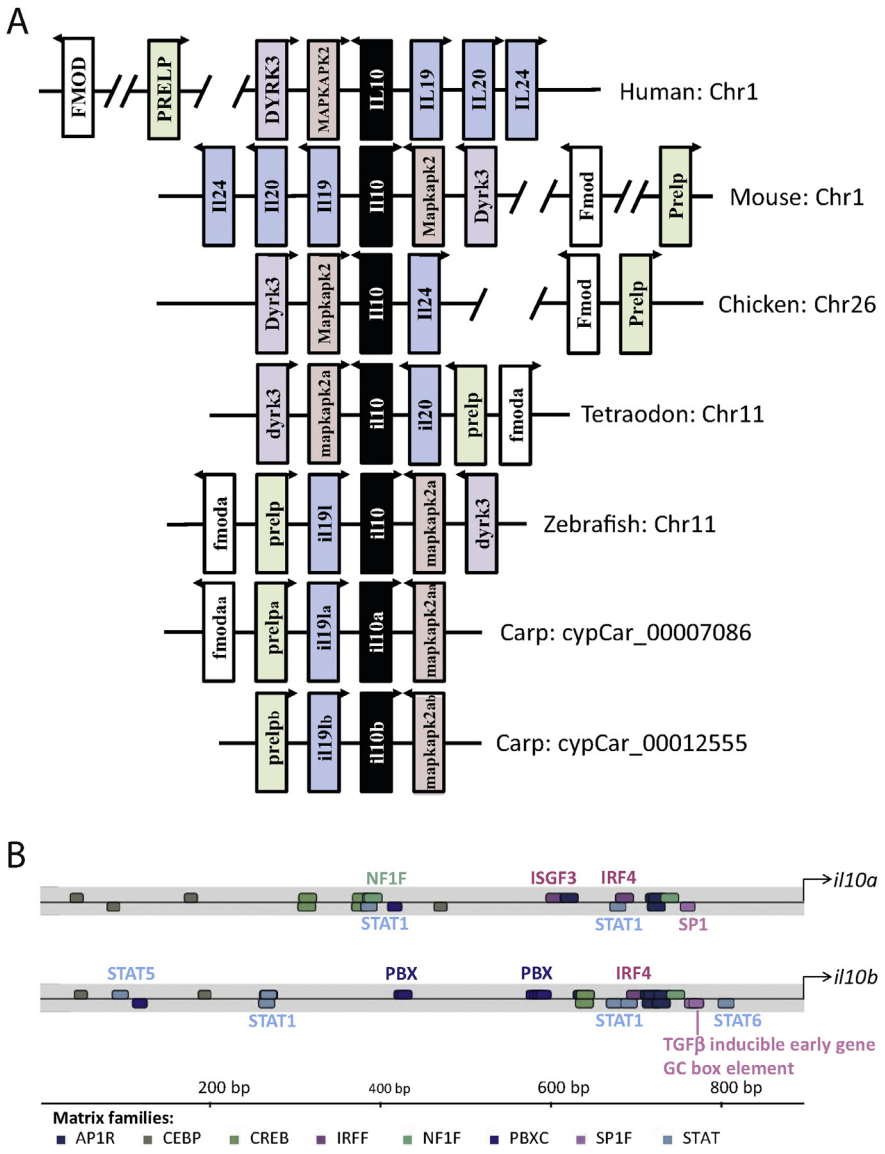

Fig. 3. While the genomic organization of both *il10* paralogues is conserved the promoter sequences are different. A) Synteny analysis of *IL10* in human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), tetraodon (*T. nigroviridis*), zebrafish (*D. rerio*) and carp (*C. carpio*) genomes. B) Promoter analysis (MatInspector) of 900 bp upstream of the start codon of Il10. From left to right: AP1R, CEBP, CREB, IRF, NF1F, PBXC, SP1F and STAT. Differences in specific transcription factor binding sites between the two paralogues are indicated.

activation of transcription of *IL10* can vary depending on the cell type and on the stimulus used (Saraiva and O'Garra, 2010). We investigated the presence of binding sites for transcription factor families previously described to bind the within the first 900 bp upstream of the ATG of the human and mouse *IL10* promoter (e.g. MAF, AP-1, CREB, IRF, NF1, PBXC, SP1F and STAT) (Mosser and Zhang, 2008; Saraiva and O'Garra, 2010). We describe important differences between the promoter sequences of the two carp *il10* paralogues, pointing to a differential regulation of the *il10a* and *il10b* genes in carp. Although the presence of most of these binding elements has also been reported for the promoter regions of other fish *il10* sequences (Seppola et al., 2008; Zou et al., 2003), indicating a conserved regulation of expression of *il10*, functional studies including different cell types and different treatments would be required to fully characterize the regulation of *il10* promotors in carp.

Because of the clear differences in the promoter regions of the two carp *il10* paralogues we expected differential regulation of expression. Indeed, the basal expression of the two carp paralogues was quite different, with *il10a* expression being higher than *il10b* in most immune tissues. Also in trout, the two *il10* paralogues are differentially expressed and regulated in different tissues (Harun et al., 2011), again with *il10a* showing the highest expression under basal conditions. Overall, tissue expression of *il10* shows a consistent pattern in teleosts, with highest expression in kidney, spleen and gills of trout, zebrafish, goldfish and carp (Grayfer et al., 2011; Inoue et al., 2005; Savan et al., 2003; Zhang et al., 2005). Our results indicate an additional, high expression of *il10* in gut and thymus and, interestingly, in liver, where *il10a* appears to be the only paralogue expressed. Immune cell types all showed some level of *il10a* and *il10b* expression, but highest expression in macrophages and neutrophils, similar to goldfish (Grayfer et al., 2011). Of interest, the expression of *il10a* in neutrophils was particularly high and is probably responsible, at least in part, for the high levels of expression found in head and mid kidney. Still, the differential expression observed in tissues cannot be entirely explained by the

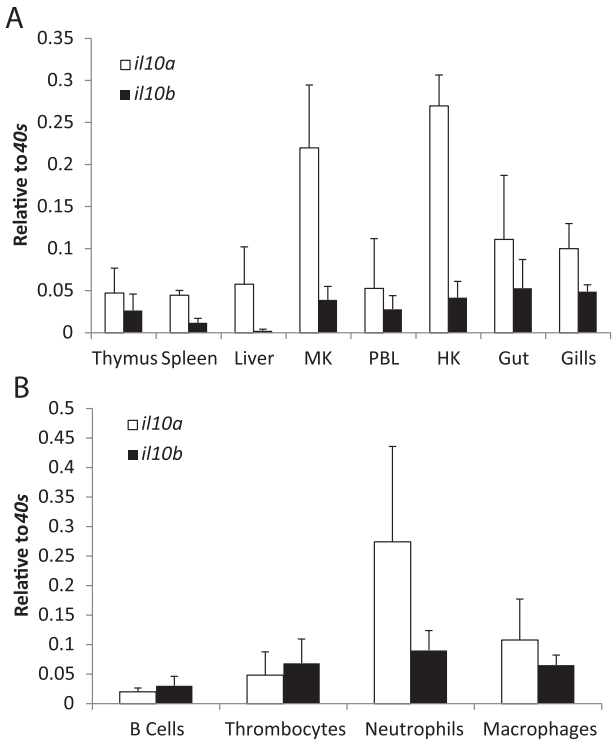

Fig. 4. Differential expression of the two *il10* paralogues. Constitutive gene expression of *il10a* and *il10b* in immune organs (A) and cell types (B). MK and HK stand for mid kidney and head kidney respectively and PBL for peripheral blood leukocytes. Basal gene expression determined by RTqPCR was normalized and expressed relative to the *s11* protein of the *40s* subunit as a reference gene. Data are presented as mean + SD of $n = 4$ fish.

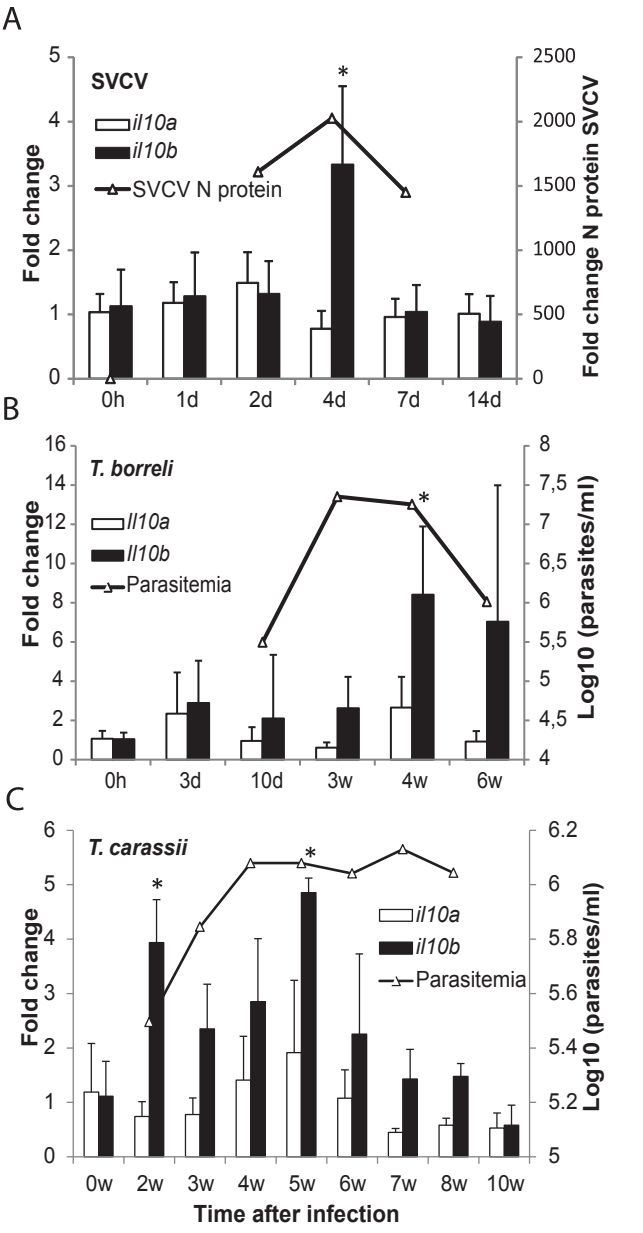

Fig. 5. Differential expression of the two *il10* paralogues upon infection. Head kidneys were collected from (A) SVCV, (B) *T. borreli* and (C) *T. carassii* infected fish. Expression of both paralogues was determined by RTqPCR and normalized relative to the *s11* protein of the *40s* subunit as a reference gene and expressed relative to uninfected fish at time point 0 h. Data are presented as mean + SD of $n = 4$ fish for SVCV and *T. carassii*, and $n = 5$ fish for *T. borreli*. Asterisks (*) indicate significant differences relative to uninfected fish.

expression observed in the selected immune cells tested. This indicates that, other immune cells and, most likely, non-immune cells can also be important sources of Il10. In the future, further studies should be conducted to specifically identify the Il10 sources in different tissues.

Trout *il10* paralogues are differentially regulated upon *Yersinia ruckeri* infection, with high expression of *il10a* in spleen and *il10b* in gills (Harun et al., 2011). We investigated differential regulation of carp *il10a* and *il10b* in head kidney upon three different infections (SVCV, *Trypanoplasma borreli* and *Trypanosoma carassii*) and found that *il10b* is the paralogue regulated in all infections, while the expression of *il10a*, which in basal conditions is much higher than *il10b*, remains constant. During SVCV infection, *il10b* was upregulated 4 days after infection, coinciding with the highest viral load and upregulation of *il12* and *ifnαβ* involved in the upregulation of a CTL response (Forlenza et al., 2008a). *T. borreli* infections induced an upregulation of *il10b* one week after the peak of parasitemia, coinciding with the upregulation of pro-inflammatory gene expression including *inos,* and subsequent increase of serum nitrite levels (Forlenza et al., 2008b). In both infection models, *il10b* expression kinetics is in line with the prototypical role of Il10, likely acting to downregulate inflammatory responses to avoid self-damage, while promoting antibody production and the development of memory cells. *T. carassii* is known to establish a chronic type of infection characterized by a mixed Th1/Th17 phenotype with the upregulation of *il17*, *il12* and *ifnγ2* at 3 weeks post infection (Ribeiro et al., 2010). During this particular infection, *il10b* showed a bimodal expression pattern, with an early peak at 2 weeks post-infection which coincided with the establishment of parasitemia and timed to occur prior to the development of an inflammatory response. This atypical early expression can be proposed as a mechanism induced by the parasite to downregulate the immune response of the host in order to successfully establish a chronic infection. Altogether, the gene expression analysis of the two *il10* paralogues clearly shows differential regulation and points at different roles for the two proteins. It would be valuable to generate an antibody able to discriminate between Il10a and Il10 b at protein level, to assess whether different transcription levels reflect differences in protein concentration and to determine whether the proteins have comparable stability (half-life) in vivo. The fact that *il10a* is highly expressed during basal conditions may indicate that this paralogue helps maintain homeostasis. Upon infection however, *il10b* would be the paralogue in charge of the

typical switch from a pro-inflammatory to an anti-inflammatory state, once the infection is under control.

Collectively, we characterized two Il10 paralogues in carp, Il10a and Il10b, with similar sequence, synteny, protein structure and bioactivity on carp innate immune cells. We report important differences in their promotor region, reflected by significant differences in their regulation upon various pathogen challenges in vivo, and their basal expression in organs and cell types. *il10a* is constitutively expressed at high level and possibly involved in the maintenance of a basal homeostatic state, whereas *il10b* is activated upon infection and probably plays the prototypical anti-inflammatory role, regulating the switch from a pro-to an anti-inflammatory response. Such clear expression diversification, possibly implying a sub-functionalization in vivo, are in line with a recent study investigating the fate of recently duplicated genes, such as those of common carp (Li et al., 2015). The analysis clearly demonstrated that slow gene loss but rapid and intense expression and function differentiation are the main driving forces of the post-WGD divergence process. In agreements, our results do not point at neo-functionalization or gene loss of one of the two paralogues rather demonstrate intense expression differentiation between the two *il10* paralogues in carp, pointing towards a sub-functionalization of the two molecules within the immune system of common carp.

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