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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,

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