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## **IL10, A Tale of an Evolutionarily Conserved Cytokine across Vertebrates**

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1 **IL10, A TALE OF AN EVOLUTIONARY CONSERVED CYTOKINE ACROSS**  
2 **VERTEBRATES**

3

4 **Running title: IL10, a tale of an evolutionary conserved cytokine**

5

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15

16 **Abstract**

17 IL10 was discovered in 1989, and since then has been the subject of intense investigation  
18 revealing its potent anti-inflammatory and regulatory activities in most immune processes  
19 during infection and disease. It was only in 2003 that the first non-mammalian *IL10*  
20 sequence was identified in teleost fish, followed in 2004 by the chicken *IL10* sequence. In  
21 this review we summarize the work performed in non-mammalian vertebrates in which the  
22 *IL10*, *IL10 receptors (IL10Rs)*, and the signaling components have been identified. We  
23 review the genomic organization, gene and protein structure of *IL10(Rs)* and focus on  
24 studies providing a functional characterization of their biological activities. In addition, we  
25 describe the activities of viral IL10s identified in viruses infecting non-mammalian hosts.  
26 Altogether, such analysis revealed a remarkable conservation of the anti-inflammatory and  
27 regulatory activities of (viral) IL10 across vertebrates, confirming the crucial role of IL10  
28 throughout evolution. Interestingly, in some teleost fish, the presence of multiple copies of  
29 *IL10(Rs)* adds an additional degree of complexity. In fact, evidence suggests that gene  
30 duplication not necessarily implies functional redundancy, leaving teleosts with additional  
31 possibilities to fine tune IL10 activities. Finally, we discuss the use of zebrafish as a  
32 complementary animal model for the study of IL10 activities in non-mammalian  
33 vertebrates.

34 **Key words**

35 (viral) Interleukin-10, Evolution, teleosts, amphibians, reptiles, birds

36

37 **Abbreviations**

38 Sp1, Sp3: Specificity protein 1 or 3, C/EBPs: CCAAT-enhancer-binding proteins, IRF-1:  
39 interferon regulatory factor 1, AP-1: activator protein 1, GATA3: GATA binding protein-3;  
40 NF1F: Nuclear factor 1F, ISGF3: Interferon-stimulated gene factor-3, PBX: Pre-B-cell  
41 leukemia transcription factor, NFAT: Nuclear factor of activated T-cells, CREBs: cAMP  
42 response element-binding. NFκB: Nuclear factor κB; (p)STAT3: (phosphorylated) Signal  
43 transducer and activator of transcription 3; JAK1: Janus kinase 1; TYK2: Tyrosine kinase  
44 2; CRFB: Cytokine receptor family B = CRF2: Cytokine receptor family class 2; LPS:  
45 lipopolysaccharide; PMA: phorbol myristate acetate; PBMC: Periferal blood mononuclear  
46 cell; PBL: Periferal blood leukocyte; BMM: Bone marrow-derived macrophages.

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70 **I. INTRODUCTION**

71 Interleukin 10 (IL10) was first discovered in 1989 upon the observation that a factor  
72 produced by mouse Th2 clones inhibited the synthesis of several cytokines by Th1 clones.<sup>1</sup>  
73 This newly discovered cytokine was first named cytokine synthesis inhibitory factor (CSIF)  
74 but the name IL10 was already applied in the follow-up publication from the same group  
75 where they described that the Epstein-Barr virus (EBV) gene BCRFI showed extensive  
76 homology with IL10.<sup>2</sup> The latter study, describing the hijacking of a host cytokine gene as a  
77 viral strategy for survival, brought to light the importance of this cytokine in immune  
78 regulation and led to a considerable amount of research describing the importance of IL10  
79 in the immune system.

80 IL10 is a pleiotropic regulatory cytokine produced by all leukocytes, with CD4<sup>+</sup> T cells and  
81 monocytes/macrophages being the most important sources.<sup>3</sup> Some non-immune cells such  
82 as keratinocytes or epithelial cells can also produce IL10.<sup>4,5</sup> The production of IL10 is  
83 tightly regulated and depends among other factors, on the stimulus, affected tissue and  
84 phase of the immune response.<sup>3</sup> The overall activity of this cytokine is to limit and  
85 terminate the immune response in order to prevent damage caused by the host's  
86 inflammatory response. Its ability to downregulate the immune response makes it a  
87 valuable target for pathogens. For example, the aforementioned EBV but also  
88 cytomegalovirus (CMV) and many more DNA viruses, have hijacked the IL10 gene into  
89 their genomes and use it to regulate the response of the host upon infection.

90 The main biological function of IL10 is exerted on dendritic cells, macrophages and  
91 neutrophilic granulocytes, inhibiting MHCII expression, differentiation of monocytes,  
92 expression of proinflammatory cytokines, phagocytosis and reactive radical species  
93 production.<sup>6,7</sup> IL10 anti-inflammatory activities are not only limited to the innate branch of

94 the immune system. It also directly inhibits proliferation of CD4<sup>+</sup> T cells,<sup>8</sup> IL2 and IFN $\gamma$   
95 synthesis by Th1 cells and IL4 and IL5 synthesis by Th2 cells.<sup>9,10</sup> The downregulation of  
96 proinflammatory activities indirectly has an effect on the resolution of the adaptive immune  
97 responses leading to an anti-inflammatory or regulatory state of immunity. IL10 has also  
98 stimulatory properties on specific cell types: it activates B cells, promotes their survival and  
99 proliferation, and contributes to class switching and antibody secretion;<sup>11,12</sup> IL10 can also  
100 stimulate NK cell proliferation and cytotoxic activity<sup>13</sup> as well as proliferation of specific  
101 subsets of CD8<sup>+</sup> T cells.<sup>14</sup> Altogether, IL10 has an important role in the termination of  
102 inflammation and restoration of homeostasis helping the development of long-lived  
103 memory cells to face future threats.

104 Based upon its structure, IL10 has been assigned to the class II helical cytokine family that  
105 includes IL10, interferons and all the so-called IL10-related cytokines (IL19, IL20, IL22,  
106 IL24, IL26, and IL28). They all share a similar overall 3D structure, are encoded by genes  
107 with a similar intron-exon structure and bind to receptors of similar structures (Class II,  
108 helical cytokine receptors) that signal through the JAK-STAT pathway.

109 IL10 acts as a homodimer that signals via the IL10 Receptor complex, constituted by two  
110 molecules of IL10 receptor 1 (IL10R1) which, upon binding to the ligand, recruit two  
111 molecules of IL10R2.<sup>15</sup> IL10R1 is specific and has high affinity for IL10 while IL10R2 can  
112 also act as co-receptor for other cytokines.<sup>7</sup> Both receptors belong to the class II cytokine  
113 receptor family (CRFB). Upon activation of the IL10R complex a JAK/STAT signaling  
114 pathway is initiated, generally triggered by the activation of JAK1 and TYK2 followed by  
115 the subsequent phosphorylation of the transcription factor STAT3. Phosphorylated STAT3  
116 stimulates the transcription of several genes, among which the *suppressor of cytokine*

117 *signaling 3 (SOCS3)*, which is considered the main transcription factor responsible for the  
118 inhibitory effects of IL10 on proinflammatory genes expression.<sup>16</sup>

119 In the past decades, the molecular structure, gene regulation, signaling pathway and  
120 bioactivity of mammalian IL10 have been extensively described and comprehensively  
121 reviewed.<sup>3,4,7</sup> Research on the biological activities of IL10 in non-mammalian vertebrates  
122 such as birds, reptiles, amphibians and fish is much more recent and scarce. In this review,  
123 keeping the activities of mammalian IL10 as reference, we aim to compile a comprehensive  
124 review of the current knowledge on this molecule in non-mammalian vertebrates. We will  
125 not only highlight the similarities between mammalian and non-mammalian IL10, but also  
126 bring to the attention of the reader the peculiarities of IL10 gene regulation, signaling  
127 pathway and bioactivities in selected non-mammalian species. In addition, we will not only  
128 review the activities of host IL10, but whenever possible, we will also include information  
129 on the bioactivities of viral IL10 identified in viruses infecting non-mammalian hosts.  
130 Finally, we will focus on the potential use of a relatively novel animal model, the zebrafish,  
131 as an additional and complementary tool for the study of non-mammalian IL10 activities.

132

### 133 **I. NON-MAMMALIAN IL10 HOMOLOGUES: CONSERVATION OF GENES AND** 134 **PROTEINS STRUCTURE**

135 IL10 sequences, both gene and protein, of hundreds of non-mammalian vertebrates can be  
136 found in the databases. Most of them are predicted sequences from the recent explosion in  
137 genome sequencing and transcriptome analysis of a large variety of species. What is clear is  
138 that IL10 homologs can be found in all vertebrate classes and their sequence is conserved to  
139 such a degree that makes it easy to identify and classify them as such (Fig. 1). Despite the  
140 abundance in gene sequences, their structural and biological characterization is somewhat

141 lacking behind. For clarity, throughout the manuscript we will adopt the human  
142 nomenclature as birds, reptiles, amphibians and teleost fish all use different nomenclatures  
143 and were often difficult to combine in a clear manner in sentences referring to genes  
144 common in all species. IL10 gene(s) and protein(s) have been well described in duck,<sup>17</sup>  
145 chicken,<sup>18</sup> frog<sup>19</sup> and several teleost fish species (fugu,<sup>20</sup> common carp,<sup>21</sup> rainbow trout,<sup>22</sup>  
146 zebrafish,<sup>23,24</sup> sea bass,<sup>25</sup> Atlantic cod,<sup>26</sup> goldfish,<sup>27</sup> Indian major carp<sup>28,29</sup> and grass carp<sup>30</sup>)  
147 (Fig. 1). Interestingly, despite several reptile *IL10* sequences can be found as predicted  
148 genes in the database (included in the phylogenetic analysis in Fig. 1) no further functional  
149 characterization of this molecule has been carried out thus far. Among the annotated, but  
150 not yet functionally characterized sequences we also find the shark, coelacanth and lungfish  
151 *IL10*-like sequences, confirming that IL10 is an evolutionary ‘old’ cytokine. Furthermore,  
152 duplicate copies of *IL10* genes have been identified in several fish species (Piazzon  
153 manuscript in preparation)<sup>31,32</sup> but not in mammals, birds, reptiles and amphibians. As it  
154 will be further discussed later, gene duplication might not always imply functional  
155 redundancy, providing teleost fish with additional tools to finely tune their IL10-mediated  
156 regulatory response.

157

#### 158 **A. Genomic and structural conservation of the *IL10* gene**

159 The synteny of the mammalian *IL10* locus is extremely conserved as in mammals the *IL10*  
160 gene is always found linked to *IL19*, and in the same relative position to *MAPKAPK2*,  
161 *DYRK3*, *PRELP* and *FMOD* (Fig. 2A). Like all *IL10* genes described in mammals, all  
162 known non-mammalian vertebrate *IL10* genes are composed of five exons and four introns  
163 (Fig. 2B). The length of exons is generally conserved and introns are in homologous  
164 positions, all in phase 0, therefore introns are not interrupting codons.<sup>25</sup> The size of the

165 introns however, varies greatly making the overall size of the *IL10* gene different among  
166 species.

167 Both, 5' and 3' untranslated regions (UTR) are also variable among species. Human and  
168 murine *IL10* cDNAs contain 7 and 6 AUUUA instability motifs respectively in the 3'UTR  
169 before the polyadenylation signal (Fig. 2B). The instability motifs are rapid RNA  
170 recognition sites for RNase E activity<sup>33</sup> important for post-transcriptional regulation of  
171 genes. In chicken<sup>18</sup> and duck<sup>17</sup> *IL10* transcripts, 11 and 6 AUUUA motifs can be observed  
172 in the 3'UTR; trout (a)<sup>22</sup> and grass carp<sup>30</sup> *IL10* transcripts present none, whereas sea bass,<sup>25</sup>  
173 common carp (a and b)<sup>21</sup> and Indian major carp<sup>29</sup> *IL10* have three. Goldfish and zebrafish  
174 possess 5 instability domains in the 3' UTR and an additional instability motif in the 5'  
175 UTR.<sup>27</sup> This differences suggest a tight regulation of this cytokine that varies greatly among  
176 species. Altogether we can conclude that the genomic organization (synteny) as well as  
177 gene organization of the *IL10* gene is highly conserved among vertebrates, further  
178 confirming the important role of this cytokine in the immune response.

179

#### 180 1. Regulatory aspects: *IL10* duplications and splice variants

181 Although not a lot of studies have been performed on the regulation of non-mammalian  
182 *IL10* and the information available is partial and fragmented, some studies have focused on  
183 interesting cases worth mentioning such as the presence of multiple copies (paralogues) of  
184 this gene or different splice variants and their possible biological implications.

185

##### 186 a. *When one is not enough! Presence of duplicated genes*

187 During evolution, after the two rounds of whole-genome duplications (WGD) that occurred  
188 in the common ancestor of vertebrates, teleost fish underwent a third duplication event<sup>34</sup>

189 implying that several genes are present in multiple copies within the fish genomes. These  
190 duplicated genes include, among many others, also cytokines, cytokine receptors and  
191 transcription factors. In addition, some fish species, including rainbow trout, Atlantic  
192 salmon or common carp, underwent an additional round of WGD<sup>35,36</sup> leading to the  
193 appearance of additional paralogues within their genome. To illustrate this complexity for  
194 the case of the *IL10* gene, rainbow trout and common carp have two paralogues (Fig. 2B),  
195 namely *IL10a* (Q6L8N7 and HQ323755) and *IL10b* (FR691804 and HQ323756),<sup>31</sup>  
196 (Piazzon, manuscript in preparation) that are very similar at the protein level but show  
197 differences in the promoter and 3' UTR, suggesting similar biological activities but  
198 differential regulation. The synteny of the paralogues is still difficult to analyze as the  
199 genome assemblies in these species are still incomplete or the scaffolds are too short, and  
200 are therefore not included in figure 2.

201 In trout, *IL10b* has a long 3'UTR with seven instability motifs, whereas *IL10a* has a short  
202 3'UTR with no instability domains identified thus far, most likely due to incomplete  
203 sequencing of the 3' UTR region. Nevertheless, gene expression analysis shows differential  
204 stability and basal expression of the two transcripts in various tissues and cell types.  
205 Interestingly, *IL10a* presents an alternative ATG in the 5'UTR that, if translated, encodes  
206 for a 29 amino acids peptide and is proposed to be a mechanism used to regulate translation  
207 of the full-length protein under certain conditions. The 5' UTR of trout *IL10b* did not  
208 extend as far, and it is still to be determined whether such regulation occurs for *IL10b* as  
209 well. As expected, the two paralogues were differentially regulated under various  
210 conditions. IFN $\gamma$  stimulation specifically affects *IL10b* expression whereas bacterial

211 infections induce differential regulation of both paralogues depending on the tissue  
212 studied.<sup>31</sup>

213 In carp, both paralogues showed similar bioactivity when tested in vitro (further discussed  
214 later) but have very different promoter regions, hinting again to a differential regulation.  
215 Carp *IL10a* is generally higher expressed in basal conditions but its expression levels do  
216 not seem to be regulated upon viral and parasitic infections. On the contrary, *IL10b* is  
217 significantly upregulated in the late phases of infection with the rhabdovirus Spring  
218 Viraemia of Carp Virus (SVCV) and the extracellular blood parasite *Trypanoplasma*  
219 *borreli* (Piazzon, manuscript in preparation) Such a differential expression pattern may  
220 confer each of the two isoforms different roles in homeostasis and pathogenesis. In  
221 agreement, a single-nucleotide polymorphism in the *IL10a* gene has been associated to  
222 resistance to cyprinid herpesvirus-3 infections<sup>32</sup> further highlighting the role of IL10 in fish  
223 immunity and disease resistance.

224 To our knowledge, the presence of duplicated copies of *IL10* (and its associated molecules)  
225 in the genome of non-mammalian vertebrates is restricted to teleost fish only, and in  
226 particular to those that underwent a 4<sup>th</sup> WGD event. Despite some amphibians, e.g.  
227 *Xenopus laevis*, are polyploid still only one IL10 gene can be found in their genome (Fig.  
228 2A), perhaps suggesting that the IL10 locus in these species is under a certain selective  
229 pressure to retain a single *IL10* copy. As expected, common carp and rainbow trout also  
230 express two copies of the *IL10 receptors*, transcription factors (*i.e.* *JAK1* and *STAT3*) as  
231 well as *SOCS3* genes. As an example, there are two paralogues of *SOCS3* in zebrafish,  
232 *SOCS3a* (NP956244) and *SOCS3b* (NP998469). Each of these genes is then present in  
233 duplicate copy in common carp and trout, adding up to a total of four *SOCS3* genes in these  
234 species. Which one of these paralogues is more important for IL10 signaling, and whether

235 these differences have any biological significance is still under investigation. What is  
236 certain is that such gene expansion greatly widens the field of study and raises the question  
237 as to whether gene duplication implies functional redundancy or sub-functionalization, as  
238 well as whether gene expansion provides an evolutionary advantage to the species. All this  
239 is currently the focus of intense research in the comparative immunology field.

240

241 *b. Post-transcriptional regulation: IL10 splice variants*

242 Splicing-derived isoforms of several cytokines and cytokine receptors have been described  
243 but poorly studied in mammals.<sup>37</sup> Regarding *IL10* very few reports exist to that respect. A  
244 new *IL10* splice variant lacking the entire exon 3, named IL10 $\delta$ 3, was described in human  
245 leukemic cells and was associated with improved response to chemotherapy.<sup>38</sup> Other  
246 authors described the presence of two splice variants in human PBMC differing in the  
247 5'UTR. One variant was constitutively expressed in unstimulated cells and contained a  
248 longer 5'UTR whereas upon stimulation with LPS the transcription of a variant with a  
249 shorter 5'UTR was induced which would have an extended half-life and be more accessible  
250 for protein translation.<sup>39</sup> Regarding viral-encoded *IL10s*, human cytomegalovirus was  
251 shown to produce several splice variants with different biological activities including the  
252 formation of complexes with human IL10 that were shown to interfere with host IL10  
253 signaling.<sup>40,41</sup>

254 Alternative splicing of the *IL10* transcript has also been described outside mammals. When  
255 performing the identification of the Pekin duck IL10, the authors described two novel *IL10*  
256 splice variants generated by exon skipping or use of an alternative exon.<sup>17</sup> Compared to the  
257 normal duck *IL10*, one of the variants showed alternative splicing in the 3'UTR region

258 leading to a different number of instability domains and stability of this transcript. The  
259 second variant presents a complete deletion of exon 5. The truncated variant retains the  
260 contact residues with the IL10R1 but lacks the F helix, possibly affecting its activity by  
261 preventing the formation of the intercalated homodimers. The basal gene expression of the  
262 truncated variant is lower but mirrors that of the wild type transcript, although its  
263 expression is not altered by stimuli that regulated wild type duck *IL10* expression. This  
264 suggests differential roles of the splice variants in homeostasis and activation. Interestingly,  
265 heterologous protein expression in human cell lines, showed that differently from the wild  
266 type protein, the truncated form was not secreted in cell culture supernatants.<sup>17</sup> Besides the  
267 aforementioned studies, no reports focused on the possible existence of splice variants of  
268 the *IL10* gene in other vertebrates. Research on the post-transcriptional regulation of *IL10*  
269 can be crucial in the understanding of the fine tuning of this potent regulatory molecule  
270 especially during pathological conditions.

271

### 272 *c. The IL10 promoter*

273 In mammals, the *IL10* promoter and the transcription of the *IL10* gene in different cell types  
274 has been studied in detail. Transcription factors such as Sp1, Sp3, STAT3, C/EBPs, IRF-1,  
275 c-Maf, AP-1, CREBs and NFκB were found to positively regulate *IL10* transcription in  
276 human and mouse and the binding site of each of these transcription factors has been  
277 mapped to specific sites in the respective promoters. All this information was extensively  
278 reviewed by Mosser and Zhang.<sup>3</sup> Despite the low sequence similarity among the promoter  
279 regions of different species, in silico comparative analysis showed several common  
280 elements in the various promoter regions. Fugu, zebrafish, cod, common carp, duck and

281 chicken *IL10* promoters present, among others, an NFκB site, interferon response elements  
282 (IREs), STAT3, GATA3, AP-1 and several Sp1 elements (Piazzon, manuscript in  
283 preparation).<sup>17,18,26</sup>

284 Interestingly, in common carp analysis of the putative promoter region of the two *IL10*  
285 paralogues showed several common binding element (e.g. for STAT1 and IRF4) but also  
286 the presence of potentially crucial differences: the *IL10a* promoter contained NF1F, ISGF3  
287 and SP1 binding sites that were not present in the *IL10b* promoter region, whereas *IL10b*  
288 had STAT6, PBX and STAT5 binding sites that were not found in the *IL10a* promoter.  
289 Altogether this could explain the differential expression of the *IL10a* and *IL10b* transcripts  
290 and suggests a potentially different function of the proteins as they are differentially  
291 regulated (Piazzon, manuscript in preparation). In mammals, the transcription factor  
292 GATA3 has been assigned a central role in activating *IL10* transcription.<sup>42,43</sup> It is also  
293 known that IL10 induces STAT3 expression and the presence of STAT3 binding sites in  
294 the IL10 promoter suggests that IL10 regulates its expression in a positive feedback loop.<sup>44</sup>

295 As a difference, while the human *IL10* promoter presents several C/EBP-β binding sites,  
296 the chicken and cod promoters only contain one, the carp promoters contains between two  
297 and four, depending on the paralogue, whereas the duck and zebrafish promoters present  
298 none.<sup>17,26</sup>

299 The presence of several common regulatory elements in the promoter regions shows that  
300 the regulation of *IL10* is somehow conserved. Nevertheless it is important to note that all  
301 above described binding sites are derived from in silico analyses and only two studies have  
302 been conducted addressing the real involvement of these transcription factors in the  
303 regulation of *IL10* transcription in non-mammalian vertebrates.<sup>29,20</sup> In Indian major carp

304 cells, the use of Bay 11-7082, a potent inhibitor of NFκB, blocked the expression of *IL10*  
305 induced by LPS suggesting that the NFκB sites found in teleost have a real regulatory  
306 function on this gene.<sup>29</sup> In fugu, the characterization of the *IL10* promoter was performed  
307 by a series of deletion mutants on the promoter region using a luciferase reporter system in  
308 trout RTG2 cell line. In this study it was shown that the binding element for NFAT,  
309 situated 92 bp upstream the TATA box, was involved in TNFα-mediated induction of *IL10*.  
310 The authors also characterized two regions in the fugu *IL10* promoter, one closer to the  
311 TATA box which would contain activating elements, and another further upstream  
312 containing inhibitory elements.<sup>20</sup> Although the study was performed in trout rather than  
313 fugu cells, it provides preliminary functional evidence of the conserved regulation of the  
314 *IL10* gene at least in teleost fish.

315 In general, little is known about the regulation of *IL10* expression in non-mammalian  
316 vertebrates. Analysis of the *IL10* promoter region in cartilaginous fish, coelacanth,  
317 amphibians and reptiles has not been conducted. Nevertheless, based on the aforementioned  
318 presence of highly conserved regulatory elements in the promoter region of the known *IL10*  
319 sequences, together with the patterns of expression in various tissues and cell types further  
320 highlighted below, it is safe to suggest that the regulation of *IL10* might be conserved  
321 across vertebrates.

322

### 323 **B. Structural conservation of the IL10 protein**

324 The IL10 proteins described in non-mammalian vertebrates range from 172 to 184 amino  
325 acids (aa) with molecular weights between 15-21 kDa, and signal peptides of 16-22 aa long.  
326 These proteins have an aa identity with their mammalian counterpart of 30-55%, with

327 *Xenopus* being the most similar, followed by birds and then fish. The degree of  
328 conservation of this cytokine among species seems low but is much higher than the  
329 interspecies conservation of other cytokines of the same structural family.<sup>45</sup>  
330 IL10 is a homodimer formed by two intertwined but non-covalently bound monomers each  
331 with six alpha-helices and two intra-chain disulphide bridges.<sup>46</sup> All the non-mammalian  
332 IL10 proteins studied present the same 6-helix structure with the four conserved cysteine  
333 residues to form the two prototypical disulphide bridges (Fig. 2C).<sup>17-19,22,23,25</sup> A single study  
334 in goldfish, using *in vitro* binding studies between recombinant IL10 and IL10R1, provided  
335 experimental evidence that also in fish IL10 might be present as non-covalently bound  
336 homodimer.<sup>47</sup> Differences in the secondary structure when compared to mammals exist but  
337 are minimal. For instance, *Xenopus* IL10 presents shorter helix A and C and longer AB and  
338 CD loops than mammalian IL10;<sup>19</sup> in sea bass the CD loop is longer than in humans and  
339 helix E is smaller;<sup>25</sup> Indian major carp IL10 has helices A and F of different length.<sup>28</sup> In  
340 general, sites and motifs essential for the bioactivity of IL10 are well preserved. The ion  
341 pair, the many hydrogen bonds and the extensive hydrophobic core to stabilize the domain  
342 structure is conserved among species. The amino acids predicted to interact with IL10R1  
343 are highly conserved or modified by similar amino acids (Fig. 2C), while the ones predicted  
344 to interact with IL10R2 are not well conserved.<sup>17-23,25-31,48</sup>  
345 The residue I69 of human IL10, key for IL10 immunostimulatory functions<sup>49</sup> can be  
346 identified in most species in a similar position and the IL10 family signature motifs are  
347 generally conserved in all investigated species.<sup>18-20,23,27,30</sup> Trout and sea bass IL10 have one  
348 potential N-glycosylation site<sup>22,25</sup>, fugu has two<sup>20</sup> and chicken and zebrafish IL10<sup>18,23</sup> have  
349 none. Human IL10 possesses one potential glycosylation site but is actually not

350 glycosylated while murine IL10 is glycosylated in its two potential sites. Nevertheless,  
351 glycosylation is not essential for IL10 bioactivity.<sup>49,50</sup>  
352 All fish IL10 present two extra conserved cysteine residues that were believed to form an  
353 additional disulphide bridge specific for fish IL10. A 3D modeling study performed on  
354 Indian major carp showed that these two cysteines do not form any significant bond  
355 involved in structural stabilization or protein-receptor interaction.<sup>28</sup> It is therefore  
356 speculated that this residues mutated during evolution in higher vertebrates.  
357 Altogether, we can conclude that across vertebrate species the structure of the IL10 protein  
358 has been extremely conserved (Fig. 2D), particularly the residues necessary for receptor-  
359 ligand interaction. As it will be further discussed below, this supports the evolutionary  
360 conservation of the regulatory functions of IL10 in non-mammalian vertebrates.

361

## 362 **II. IL10 RECEPTORS AND SIGNALING PATHWAY**

363 IL10 exerts its functions upon binding to the IL10 receptor complex on the cell surface. The  
364 IL10 receptor complex is constituted by two class II cytokine receptor (CRF2 or CRFB)  
365 family members, one belonging to the R1 type with a long intracellular domain (IL10R1 or  
366 CRFB7 in fish), and the other to the R2 type with a short intracellular domain (IL10R2 or  
367 CRFB4 in fish) (Fig. 3A).<sup>51,52</sup> Binding of the IL10 homodimer to two IL10R1 molecules  
368 induces a conformational change in the cytokine allowing the association of two IL10R2  
369 molecules.<sup>53</sup> The latter activates the Janus kinases Jak1 and Tyk2 associated with the  
370 cytoplasmic tails of IL10R1 and IL10R2 respectively.<sup>54,55</sup> All this leads to phosphorylation  
371 of STAT3 or other latent transcription factors depending on the cell type.<sup>56,57</sup>  
372 The components of the IL10 signaling pathway have been well characterized in mammals  
373 and there are only a few studies dealing with their functional characterization in non-

374 mammalian vertebrates. While IL10 genes have been easily identified using whole genome  
375 sequences, identification of its receptor chain in non-mammalian vertebrates, in particular  
376 in teleost fish, has been more challenging due to higher sequence divergence. Based upon  
377 the first high quality whole genome sequences from fish species, a repertoire of genes  
378 encoding class II helical cytokine receptors in fish has been established. They have been  
379 called CRFB1 to CRFB17 (Fig. 3B).<sup>58,59</sup> Due to high sequence divergence, sequence  
380 similarities are not a sufficient criterion to assign a function to most of these CRFBs in fish.  
381 Furthermore, some fish species (e.g. common carp, rainbow trout, and Atlantic salmon)  
382 have duplicated copies of some of these genes (Fig. 3C). Additional criteria such as synteny  
383 may be used, but functional identification based upon biological activity in at least one fish  
384 species is necessary.

385

#### 386 **A. The IL10 receptor 1**

387 Several *IL10R1* sequences (such as those for chicken (AM049243), turkey  
388 (XP\_003212786), finch (XP\_002189322), Chinese softshell turtle  
389 (ENSPSIG00000002111) and frog (XP\_002932948)) can be found in the databases as  
390 automatic predictions and genome annotations. Functional studies on non-mammalian  
391 species were performed only very recently in Pekin duck<sup>52</sup>, zebrafish<sup>47</sup>, and goldfish,<sup>47</sup> in  
392 fish, CRFB7 was identified as being *IL10R1*. Compared to their ligand, the *IL10R1*  
393 sequences have diverged to a larger degree throughout evolution. Nevertheless, the  
394 genomic organization (synteny) and gene structure of the CRFB family members that  
395 include the *IL10R1* homologues is highly conserved (Fig. 4) and allowed for a relatively  
396 straightforward identification of the IL10R1 (CRFB7) in non-mammalian vertebrates.

397 In the human, chicken, frog and zebrafish genomes the gene(s) is always flanked by  
398 *TMPRSS13*, *FXVD6* and *DSCAML1* upstream, and by *TMPRSS4*, *SCN4B* and *SCN2B*  
399 downstream (Fig. 4A) making it easier to identify the *IL10R1* sequences by synteny  
400 analysis.<sup>47</sup> Like most other class II helical cytokine receptors in vertebrates, IL10R1  
401 (CRFB7) is encoded by a gene composed of 7 exons and 6 introns of respective phases 1, 2,  
402 1, 0, 1 and 0 (Fig. 4B).

403 The mammalian, avian, amphibian and fish *IL10R1* genes encode proteins with conserved  
404 residues particularly in the regions that are needed for the formation of the hydrophobic  
405 patches where ligand binding occurs.<sup>47,52</sup> With respect to the intracellular portion of the  
406 receptor, JAK1-binding motive (PXXL) has been highly conserved and can be found within  
407 the first cytoplasmic residues in all species studied (Fig. 4C).<sup>47,52</sup> Two conserved peptide  
408 motifs containing a conserved tyrosine residue (GYXXQ) predicted to be involved in the  
409 recruitment of STAT3 can be found after the JAK1 binding site in avian<sup>52</sup> and most  
410 mammalian sequences (Fig. 4C). In some mammalian species such as mouse and rat as well  
411 as in birds, an additional STAT3 recruitment site can be found upstream of the canonical  
412 sites. Fish and frog sequences present one very conserved STAT3 recruitment site,<sup>47</sup>  
413 identified as GYXXQ, and a second non-canonical site identified as DYLLQ in frog and  
414 GYRSG in fish. In fish and birds but also in rabbit and horse a third tyrosine residue can be  
415 found downstream of the other two (canonical) STAT3 recruitment sites (Fig. 4C). Finally,  
416 in fish species where the ligand was found to be duplicated (e.g. common carp) also the  
417 receptor is present in two copies, adding an additional degree of complexity to the  
418 understanding of IL10 signaling in fish. A report in rainbow trout described one CRFB7  
419 molecules,<sup>60</sup> but this might also be due to the preliminary assembly of the genome.  
420 Furthermore, the exact contribution of each of the canonical as well as additional (potential)

421 STAT3 recruitment sites in the cytoplasmic tail of the IL10R1 of fish and frog has not been  
422 systematically addressed and awaits further investigation.

423 IL10R1 is typically expressed on immune cells and in immune organs.<sup>4</sup> Avian and fish  
424 IL10R1 are most expressed in spleen and thymus followed by bursa, lung and cecal tonsil  
425 in the case of birds and gills, kidney and gut in fish.<sup>47,52,61</sup> In general, highest expression is  
426 detected in hematopoietic (fish kidney, avian bursa) and immune organs, especially in  
427 mucosal immune tissues such as gut, lung and gills. In carp and goldfish, IL10R1 is highest  
428 expressed in macrophages, considered the main cellular target of IL10, followed by  
429 neutrophils, B cells and thymocytes.<sup>47,61</sup> In goldfish monocytes, IL10R1 is specifically  
430 downregulated by inflammatory signals, such as bacterial or parasite antigens, but is  
431 marginally regulated by poly I:C or zymosan.<sup>47</sup> Duck PBMCs stimulated with PMA exhibit  
432 a rapid upregulation of the receptor in the first 2 hours, falling even below the basal levels  
433 after 8 hour stimulation.<sup>52</sup> Not much more is known about the regulation of the expression  
434 of IL10R1 besides mammals. The data so far indicate that inflammatory stimuli would  
435 generally downregulate the expression of this anti-inflammatory-related molecule and that  
436 IL10 itself is also able to downregulate its own receptor, hinting at a conserved negative  
437 feedback loop in the IL10 system.<sup>47</sup>

438

#### 439 **B. The IL10 receptor 2**

440 The IL10 receptor 2 belongs to the R2 type subunits of CRFB receptors. These subunits  
441 contain shorter intracellular domains and act as co-receptors for the R1 subunits after  
442 binding of the latter to the ligand.<sup>62</sup> IL10R2 is not exclusive for IL10 and, in mammals, also  
443 serves as a co-receptor for other IL10 family members (i.e. IL22, IL26) and for type III  
444 interferon signaling.<sup>63-65</sup> In mammals, the gene cluster *IFN- $\alpha$  receptor-2 (IFNAR2)*,

445 *IL10R2*, *IFNAR1* is a very conserved group of synteny.<sup>66</sup> The first non-mammalian *IL10R2*  
446 sequence was identified in chicken using a hybridization probe against human *IL10R2*; by  
447 synteny analysis it led to the identification of *IFNAR1* and *IFNAR2* (Fig. 5A).<sup>51</sup> In the same  
448 study, the hybridization approach failed to identify the *IL10R2* gene in a fish genome.  
449 Owing to the first available high quality fish genomes, and using protein similarities, gene  
450 structure similarities and synteny, the fish homologues of the mammalian *IL10R2* gene  
451 have been identified in 2003.<sup>58</sup> They are named *CRFB4* and *CRFB5* and are present in all  
452 fish genomes analyzed so far. They are most probably derived from a recent duplication  
453 event. As it can be easily appreciated in figure 5A, the gene cluster *IFNAR2*, *IL10R2*,  
454 *IFNAR1* is highly conserved not only in mammals, but also in birds, reptiles and  
455 amphibians. Such conservation however is completely lost when it comes to fish genomes  
456 (Fig. 5A);<sup>67</sup> also when comparing several fish genomes, many differences can be found in  
457 the locus organization of most of the *CRFBs* homologous to the genes involved in these  
458 gene cluster. For example, all fish express two *IFNAR2* homologues named *CRFB1* and  
459 *CRFB2* (Fig. 5A)<sup>68</sup> but they are often found in regions very distant from, rather than in  
460 proximity of, the putative *IL10R2* genes (i.e. *CRFB4* and *CRFB5*). Furthermore, a fish-  
461 specific *CRFB3* gene is present only in some fish species, but when present, it is found in  
462 the gene cluster neighboring the potential *IL10R2* genes. To complicate matters, the *CRFB6*  
463 gene (previously confirmed to be the *IFNGR2* homologue)<sup>59</sup> is present in all fish species,  
464 but only in some it is found neighboring the *CRFB4* or *CRFB5* gene; similarly to *CRFB3*,  
465 *CRFB4* and *CRFB5*, it encodes a protein with a short cytoplasmic tail. Altogether, solely  
466 based on *CRFB4* and *CRFB5* protein structure (both encoding for a co-receptor with short  
467 cytoplasmic tail), or on the genomic organization of the locus, it was not possible to  
468 unequivocally determine which of the two would be the functional equivalent of *IL10R2*.

469 The question about which between CRFB4 and CRFB5 could act as the actual co-receptor  
470 of IL10R1 (CRFB7) was recently addressed in grass carp using a functional approach.  
471 Grass carp kidney cell lines were transfected with a pSTAT3-luciferase reporter plasmid  
472 together with a vector encoding for the IL10R1 in combination with either CRFB4 or  
473 CRFB5. After stimulation with recombinant IL10 an increase in the luciferase activity was  
474 observed only in cells transfected with the CRFB7+CRFB4 combination, providing the first  
475 functional indication that CRFB4 is the likely co-receptor for the IL10R complex in fish.<sup>69</sup>  
476 Previous functional studies in zebrafish on the characterization of the type I IFN receptor  
477 complex indicate that CRFB5 acts as the co-receptor for CRFB1 and CRFB2 involved in  
478 type I IFN signaling.<sup>59</sup> Based on the functional work performed in grass carp and zebrafish,  
479 and despite the high sequence similarity between CRFB4 and CRFB5, it is unlikely that the  
480 type I IFN and the IL10 system would share common co-receptor subunits. This leaves  
481 indeed CRFB4 as the most likely co-receptor of CRFB7 in IL10 signaling. Nevertheless,  
482 only a systematic functional approach using both, IL10 and type I IFN ligands would give  
483 us a definite answer.

484 It is very important to mention that in databases such as ensembl.org automatic gene  
485 annotation assigned the name *IL10R2* (or *IL10Rb*) to all *CRFB5* present in fish genomes.  
486 When the *IL10R2* GeneTree is generated in ensembl  
487 (<http://www.ensembl.org/Multi/GeneTree/Image?gt=ENSGT00530000063449>) two main  
488 clusters are clearly generated: one containing the *IFNARI* sequences clustering together  
489 with the fish *CRFB5* (here wrongly named *IL10R2/b*) and a second branch containing fish  
490 *CRFB4* grouping together with the *IL10R2* sequences in other species. Therefore, in this  
491 example, phylogenetic analyses already hint at the incorrect annotations of the *CRFB4* and

492 *CRFB5* sequences in the database, and stress the confusion that can be generated by  
493 automated annotations.

494 With respect to gene structure and expression, in all investigated vertebrates, including fish  
495 *CRFB4* and *CRFB5*, the genes present seven exons of conserved length and six introns of  
496 variable length (Fig. 5B). Like in mammals, gene expression studies in frog and duck  
497 (*IL10R2*) and fish (*CRFB4* and *CRFB5*) show that these molecules are constitutively  
498 expressed in all tissues examined being highest expressed in immune organs and lowest in  
499 muscle, heart and brain.<sup>58,69-71</sup> The expression levels remain stable in most cells even after  
500 activation.<sup>65,71</sup> With respect to protein structure, chicken, duck and frog *IL10R2* genes  
501 encode for proteins that have about 40% amino acid identity to the human counterpart,  
502 while fish proteins are only 30% identical to the human homologue. *IL10R2* proteins from  
503 fish and amphibian share the 4 conserved cysteine residues important for the linkage of the  
504 extracellular  $\beta$ -strands, but chicken and duck proteins only present 3 of these 4 conserved  
505 residues.<sup>70</sup>

506 Altogether, in teleost fish *CRFB* genes have evolved rapidly and independently not only  
507 from their mammalian counterpart but also from homologous genes in other tetrapods. This  
508 is especially reflected in the poor conservation of the *IFNAR2*, *IL10R2*, *IFNAR1* genomic  
509 locus in teleosts. The approach taken to identify *CRFB4* as the functional *IL10R2*, shows  
510 how functional analysis, together with genomic and gene structure analysis, have all been  
511 instrumental to unravel the role especially of this fast evolving gene. The incorrect  
512 annotation in the database of *CRFB5* as *IL10R2*, further confirms how automated analysis,  
513 not supported by functional data, can lead to incorrect conclusions. Finally, considering that  
514 in some species, such as common carp (unpublished observation), Atlantic salmon<sup>72</sup> and

515 possibly trout, the genes encoding for IL10 and its receptors are duplicated, we can expect  
516 that unique features and regulatory mechanisms might be unraveled by the study of  
517 duplicated genes in teleost fish.

518

### 519 **C. Downstream signaling**

520 In mammals, upon binding of IL10, the IL10 receptor complex activates the Janus tyrosine  
521 kinases, JAK1 and TYK2, associated with IL10R1 and IL10R2 respectively. The  
522 cytoplasmic tail of IL10R1 is phosphorylated leading to the recruitment and subsequent  
523 phosphorylation of STAT3 by the kinases.<sup>3</sup> What happens downstream the IL10 receptor is  
524 not very well documented in non-mammalian species, with only a few reports in fish  
525 dealing with the prototypical signaling cascade of STAT3 phosphorylation and activation  
526 of the *SOCS3* gene. By use of cross-reacting antibodies recognizing phosphorylated  
527 STAT3, it was possible to show that goldfish, common carp and grass carp IL10 induce  
528 STAT3 phosphorylation and translocation to the nucleus.<sup>27,30,61</sup> Phosphorylation of  
529 cytoplasmic STAT3 occurs in the first 15 minutes after stimulation even though the cellular  
530 association of IL10 with the receptor persists for more than 90 minutes.<sup>27</sup> *SOCS3*  
531 expression, in fish as in mammals, is also upregulated within the first hours of exposure to  
532 IL10<sup>27,30,61</sup> and this effect can be abolished by a STAT3 inhibitor.<sup>30</sup> What remains to be  
533 studied, in fish and in other vertebrate species, is the significance of the various canonical  
534 and non-canonical STAT3 binding sites and how this, together with serine-rich stretches of  
535 residues, might affect the downstream signaling. Furthermore, in human and mice it has  
536 been observed that not all STAT3-inducing receptors, e.g. IL6R, trigger anti-inflammatory  
537 responses. This implies that activation of STAT3 might not be the only mechanism  
538 required for the anti-inflammatory activity of IL10. Inhibition of NFκB activation,

539 translocation as well as DNA binding have all been shown to occur in various cell types  
540 following IL10 stimulation. The inhibition of NFκB activity by IL10 would explain the  
541 large number of immune response genes that are less responsive to stimuli or are  
542 downregulated following IL10 treatment (reviewed by Mosser and Zhang).<sup>3</sup> SOCS3  
543 activation is a hallmark of IL10 (and not IL6) induced gene expression, possibly suggesting  
544 that SOCS3 might play a unique role in the IL10-specific response. Detailed analysis of the  
545 IL10 signaling pathway in various cell types, besides the activation of STAT3, has not been  
546 systematically addressed in non-mammalian species. As mentioned above, the cytoplasmic  
547 tail of the IL10R1 presents various canonical and non-canonical STAT3-binding sites. This,  
548 together with the presence of additional tyrosine and serine-rich motives in the cytoplasmic  
549 tail, leaves open the possibility that, also in non-mammalian species, IL10 might act  
550 through signaling mechanisms other than STAT3.

551

### 552 **III. BIOACTIVITY**

553 The conservation of the IL10 protein, signaling pathway and the expression dynamics upon  
554 stimulation or infection hint to a conservation of bioactivity when compared to mammals.  
555 But actual bioactivity studies of non-mammalian IL10 on different cells of the immune  
556 system are very scarce and have been conducted only in a few avian and fish species.

557

#### 558 **A. Bioactivity on phagocytes**

559 Monocytes, macrophages and neutrophilic granulocytes are among the main targets of  
560 IL10. This cytokine is known to strongly inhibit phagocytes by downregulating the  
561 production of toxic radicals, phagocytosis, antigen presentation and expression of  
562 proinflammatory cytokines.<sup>3,6</sup>

563 The only study in chicken addressing the inhibitory activity of IL10 on macrophages made  
564 use of neutralizing antibodies against chicken IL10.<sup>73</sup> Chicken bone marrow-derived  
565 macrophages (BMMs) were shown to produce nitrogen radicals upon LPS stimulation and  
566 to readily express IL10 protein as early as 2 h after stimulation. Under the same conditions,  
567 the addition of IL10 neutralizing antibodies led to a significant increase in nitrogen radical  
568 production by BMMs.

569 The effect of IL10 on phagocytes of other avian species, reptiles or amphibians has not  
570 been investigated thus far. Nevertheless, a substantial amount of data is available from  
571 studies in various teleost fish species. Recombinant goldfish IL10 was shown to  
572 significantly reduce the respiratory burst induced in goldfish monocytes by *Aeromonas*  
573 *salmonicida* or IFN $\gamma$  stimulation as well as the expression of several pro-inflammatory  
574 genes including *TNF $\alpha$ 1*, *TNF $\alpha$ 2*, *IL10*, *CXCL8* and the NADPH oxidase component  
575 *p47<sup>phox</sup>*. Under the same conditions, goldfish splenocytes showed downregulation of the  
576 expression of IFN $\gamma$ .<sup>27</sup> In mammals, the inhibition of the respiratory burst in macrophages by  
577 IL10 is mainly attributed to an indirect effect of IL10 acting through the downregulation of  
578 TNF $\alpha$  rather than directly on radical production and release.<sup>74,75</sup> In the case of goldfish,  
579 besides downregulation of *TNF $\alpha$ 1* and *TNF $\alpha$ 2*, a direct effect of IL10 on the respiratory  
580 burst was demonstrated due to the direct downregulation of NADPH oxidase components.<sup>27</sup>  
581 Recombinant carp IL10, similarly to goldfish IL10, significantly inhibited the PMA and  
582 LPS induced production of toxic oxygen and nitrogen radicals in carp macrophages and  
583 neutrophils.<sup>61,76</sup> The effect was dose dependent and very rapid, again pointing towards a  
584 direct inhibitory effect of IL10 on fish phagocytes. Carp IL10 also inhibited the LPS-  
585 induced expression of proinflammatory genes in macrophages and neutrophils. More

586 specifically, *IL1 $\beta$* , *TNF $\alpha$* , *iNOS* and *IL6* were downregulated in both cell types and the *p35*  
587 gene was downregulated only in macrophages. Carp IL10 also showed inhibitory effects on  
588 genes involved in antigen presentation in carp neutrophils, but not macrophages, as it  
589 downregulated the expression of *MHCI* and *MHCII* genes<sup>61</sup> and the surface expression of  
590 MHCII protein.<sup>76</sup> Interestingly, as mentioned above, common carp and trout present two  
591 copies of the *IL10* gene both encoding for potentially functional proteins. While the  
592 biological activity of both isoforms was not compared in trout, functional studies in  
593 common carp, using recombinant IL10a and IL10b, clearly indicate that the two proteins  
594 have identical biological activities. Nevertheless, as discussed in section I.A.1.a., the  
595 transcriptional regulation of the paralogues is different under various conditions,  
596 consistently with their different promoter regions. This indicates that although they might  
597 bind to the same receptor complex and trigger the same signaling in carp leukocytes, they  
598 might not be expressed under the same circumstances and at the same level. This points  
599 towards a possible sub-functionalization, rather than functional redundancy, of paralogous  
600 genes, further increasing the level of regulation and fine tuning of the immune system in  
601 those species presenting multiple gene copies.

602 The direct effect of fish IL10 on phagocytes was also studied in grass carp. Recombinant  
603 grass carp IL10 inhibits the LPS-induced transcription of *TNF $\alpha$* , *IL1 $\beta$* , *IL8* and *iNOS* in  
604 monocytes/macrophages.<sup>77</sup> On the same cells the authors also tested the effect that  
605 endogenous IL10 had on TGF $\beta$ 1 expression, another important regulatory cytokine. LPS  
606 was found to induce proinflammatory gene expression in monocytes/macrophages after 6 h  
607 and the upregulation was reduced at 12 h when endogenous IL10 and TGF $\beta$ 1 mRNA and  
608 protein levels increased. When IL10 and TGF $\beta$ 1 blocking antibodies were used, the

609 stimulatory effects of LPS were still significantly high at 12 h, confirming the inhibitory  
610 activity exerted by the endogenously produced anti-inflammatory cytokines. The inhibitory  
611 activity exerted by grass carp IL10 and TGF $\beta$ 1 on LPS-induced NF $\kappa$ B activation was also  
612 investigated. The protein I $\kappa$ B $\alpha$ , which inhibits NF $\kappa$ B by blocking its ability to bind DNA,  
613 is degraded in grass carp monocytes/macrophages upon LPS stimulation. Both, IL10 and  
614 TGF $\beta$ 1 showed the ability to block LPS-induced I $\kappa$ B $\alpha$  protein degradation thereby  
615 attenuating the pro-inflammatory effect of LPS.<sup>77</sup>

616 Altogether we can conclude that the prototypical anti-inflammatory activities of IL10 on  
617 phagocytes are generally conserved also in non-mammalian vertebrates. What perhaps still  
618 needs to be further investigated is the ability of IL10 to also inhibit antigen presentation by  
619 macrophages. The studies performed so far in fish on the regulation of antigen presentation  
620 do not show a significant effect of IL10 on macrophages.<sup>78</sup> The study however only  
621 focused on *MHCII* transcription rather than protein expression, leaving open the possibility  
622 that IL10 might directly affect MHCII protein expression on macrophages thereby lowering  
623 their antigen presentation capacity.

624

## 625 **B. Bioactivity on lymphocytes**

626 The effect of IL10 on B and T lymphocytes is diverse. On the one hand IL10 is known to  
627 induce proliferation, antigen presentation, differentiation and antibody secretion in B  
628 lymphocytes<sup>11,12</sup> and to promote proliferation of subsets of CD8<sup>+</sup> T lymphocytes.<sup>14</sup> On the  
629 other hand, it directly inhibits cytokine synthesis and proliferation of CD4<sup>+</sup> Th1 and Th2  
630 lymphocytes, indirectly affecting the progression or the resolution of the adaptive immune  
631 responses.<sup>8-10</sup> The paucity of tools available to study B and T cell biology in non-

632 mammalian vertebrates makes the characterization of these cells and their function very  
633 difficult. Only few markers are available to separate different cell populations and the  
634 different lymphocyte responses known in mammals have not been fully characterized in all  
635 non-mammalian vertebrate species. Nevertheless, some advances have been made in the  
636 last years, especially in chicken and in a few teleost fish species owing to the development  
637 of B and T cell-specific monoclonal antibodies or to the identification of cross-reactive  
638 antibodies against mammalian transcription factors.

639 In chicken, recombinant IL10 inhibits IFN $\gamma$  transcription and protein expression in mitogen  
640 stimulated lymphocytes from spleen. IL10 also inhibits the ability of the supernatants of  
641 these stimulated lymphocytes to induce nitrogen radicals, probably due to the lower  
642 concentrations of IFN $\gamma$ , indirectly affecting the activity of the phagocytes.<sup>18</sup> Duck  
643 recombinant IL10 inhibits the expression of IL2 induced by mitogen stimulation of  
644 PBMCs.<sup>17</sup>

645 In teleost fish, recombinant carp IL10 inhibited the IL2-induced proliferation of  
646 thymocytes.<sup>76</sup> This is in contrast with the activity of mammalian IL10 on the same cell  
647 type<sup>79</sup> but the biological implications of this difference remain to be studied. Interestingly,  
648 only in immunized carp, IL10 showed to enhance proliferation of a subpopulation of T cells  
649 when administered with the immunizing antigen.<sup>61</sup> Under the same conditions IL10 had no  
650 effect on proliferation of naïve T cells, suggesting that the stimulatory effect of IL10 is  
651 restricted to a subpopulation of memory T cells. Due to the lack of antibodies against T  
652 cell surface markers, the class of T cells involved in this response was characterized only  
653 by real time-quantitative PCR and the results indicated that IL10 inhibited the Th1 and Th2  
654 responses induced by the immunizing antigen while promoting the proliferation of a subset

655 of CD8<sup>+</sup> T cells. Further characterization of the specific T cells populations stimulated by  
656 IL10 is expected soon owing to newly developed antibodies specific for various subsets of  
657 carp T cells.

658 In carp, the availability of an anti-IgM antibody<sup>80</sup> allowed for the study of the effect of  
659 IL10 specifically on IgM<sup>+</sup> B cells. Recombinant carp IL10 directly promoted IgM<sup>+</sup> B cell  
660 proliferation in sorted cells and in mixed PBL cultures; the stimulatory effect was further  
661 enhanced by LPS or *Trypanoplasma borreli* antigens, both known to induce a polyclonal  
662 activation of carp IgM<sup>+</sup> B cells.<sup>61,76</sup> Contrary to what was found in neutrophils, IL10  
663 increased the surface expression of MHCI molecules in IgM<sup>+</sup> B cells possibly improving  
664 antigen presentation by these cells.<sup>76</sup> Regretfully, the lack of specific antibodies to detect  
665 MHCII left this characterization incomplete, but what is clear is that carp IL10 exerts  
666 differential and cell type-specific effects on MHCI protein expression with possible  
667 consequences on antigen presentation. In carp head kidney leukocyte cultures IL10 induced  
668 an increase in secreted total and antigen specific IgM, which also correlated with an  
669 increase in differentiation of plasmablasts to plasma cells.<sup>61</sup> These studies in carp show well  
670 conserved bioactivity of IL10 on B cells when compared to mammals but focus only on  
671 IgM<sup>+</sup> B cells. To complete these studies, the effect of fish IL10 on IgT<sup>+</sup> and IgD<sup>+</sup> B cells  
672 should be conducted.

673 In grass carp IL10 enhanced cell viability of PBLs. Although the specific cell type affected  
674 was not characterized, this activity resembled the prototypical effect of IL10 on B cells.  
675 This effect was also shown upon incubation with TGFβ1, but further studies using blocking  
676 antibodies against IL10 and TGFβ1, confirmed that the actual induction of proliferation is  
677 due to the endogenous IL10 whose expression and secretion is activated by the TGFβ1.<sup>30</sup>

678 In general, besides the work performed in fish, in particular in common carp, not much is  
679 known about the activities of IL10 on lymphocytes in non-mammalian species.  
680 Nevertheless, based on the work in teleost fish, on the indirect data in chicken and duck,  
681 and considering the structural conservation of the protein discussed above, it could be safe  
682 to speculate that IL10 activities on lymphocytes might be conserved also in non-  
683 mammalian vertebrates.

684

#### 685 **IV. VIRAL HOMOLOGS**

686 A common strategy used by DNA viruses to counteract the host immune system is the  
687 expression of homologs of host genes, in particular cytokines, chemokines, growth factors  
688 and cytokine receptors.<sup>81</sup> IL10 homologs have been identified in multiple members of the  
689 Poxviridae and Herpesvirales and, although they share relatively low amino acid identity  
690 with their host counterpart, they can still bind to the IL10R complex, effectively mimicking  
691 at least part of the biological activities of the host protein.<sup>82,83</sup> Among the most studied  
692 IL10 viral homologs are those produced by the human Cytomegalovirus (CMV)<sup>84</sup> and  
693 Epstein-Barr virus (EBV),<sup>2</sup> although more than 20 cytokine homologs have been described  
694 in viruses infecting mammals including horse,<sup>85</sup> monkeys,<sup>86</sup> sheep,<sup>87,88</sup> cow,<sup>89</sup> goat,<sup>90</sup>  
695 camel<sup>91</sup> and even bats.<sup>92</sup> This phenomenon is not restricted to mammals, as several viruses  
696 infecting birds (pigeon pox virus, penguin pox virus<sup>93</sup> and canary pox virus),<sup>94</sup> reptiles  
697 (testudinid herpesvirus)<sup>95</sup> and fish (anguillid herpesvirus 1<sup>96</sup> and cyprinid herpesvirus 3)<sup>97</sup>  
698 present IL10 homologs in their genomes. Sequence analysis of these homologs showed  
699 again low sequence identity but conservation of the essential residues required for receptor-  
700 binding. Nevertheless, uncharacterized biological functions for these proteins cannot be  
701 excluded. Besides studies on CMVIL10 and EBVIL10, functional studies on the biological

702 activities of viral cytokine homologues have been conducted only on the cyprinid  
703 herpesvirus 3 IL10 homologue ( $CyHV3IL10$ ).

704 Open Reading Frame 134 (ORF134) of  $CyHV3$  encodes for the  $CyHV3IL10$ , which was  
705 shown to be the second most abundant protein in the virus secretome.<sup>98</sup> It was found to be  
706 highly expressed in infected carp tissues during the acute and reactivation phases of viral  
707 infection and at lower levels during virus persistence at low temperatures.<sup>99</sup> The predicted  
708 three-dimensional structure and residues important for the interaction with the IL10R1 are  
709 highly conserved.<sup>48</sup> Indirect evidence of  $CyHV3IL10$  signaling via this receptor was provided  
710 by a study in zebrafish using a morpholino approach, in which knock-down of the *IL10R1*  
711 abrogated the response to both  $CyHV3IL10$  and zebrafish IL10.<sup>99</sup> More direct evidence was  
712 provided by work in common carp, in which recombinant  $CyHV3IL10$  was shown to induce  
713 phosphorylation of STAT3 and expression of *SOCS3* in carp leukocytes.<sup>76</sup> Furthermore,  
714 recombinant  $CyHV3IL10$  was shown to share several activities with its host counterpart, carp  
715 IL10: it inhibited the respiratory burst in phagocytes, downregulated the expression of  
716 proinflammatory genes in macrophages and promoted proliferation of IgM<sup>+</sup> B cells and of  
717 certain subsets of memory CD8<sup>+</sup> T cells.<sup>76</sup> In zebrafish, injections of  $CyHV3IL10$  mRNA  
718 induced an increase in the number of lysozyme-positive cells in zebrafish embryos in a  
719 manner similar to zebrafish IL10.<sup>99</sup> Nevertheless, similarly to some mammalian viral  
720 cytokines such as  $EBVIL10$ , it does not mimic the full repertoire of host IL10 activities.  
721  $CyHV3IL10$  presented lower effects on the inhibition of proinflammatory cytokines  
722 expression in neutrophils, failed to inhibit nitrogen radical production and did not affect  
723 expression of molecules involved in antigen presentation and thymocyte proliferation.<sup>76</sup>  
724 These differences are most likely due to difference in affinity of the viral IL10 to the  
725 receptor, but the possibility of an alternative signaling pathway, depending on the cell type,

726 cannot be excluded. Some effects of CyHV3 infections on the innate immune response of  
727 the host, such as inhibition of type I interferons<sup>100</sup> and inhibition of apoptosis,<sup>101</sup> have also  
728 been attributed to its ability to express an IL10 homolog among other anti-inflammatory  
729 proteins. Interestingly, although *CyHV3*IL10 is highly secreted upon infection and has  
730 important anti-inflammatory properties, in vivo studies using recombinant virus strains with  
731 a deleted ORF134, suggested that *CyHV3*IL10 is not essential for viral replication in vitro or  
732 virulence in vivo.<sup>98</sup> This apparent contrast should be further studied to unravel the  
733 biological relevance of this viral homolog. Considering the importance of IL10 in  
734 regulating the immune response and the vast number of viruses carrying IL10 homologues,  
735 it would be certainly interesting to gather more data on the function of viral IL10s in other  
736 non-mammalian species. Furthermore, considering the different environments and body  
737 temperature that the various hosts live in, it would be interesting to investigate how and  
738 possibly why the same viral IL10 homologue has been retained throughout viral evolution.  
739 This will not only give important insight in virus biology, but will certainly help us  
740 understand the key features of the host IL10 that have been retained through host and virus  
741 evolution.

742

## 743 **V. IL10 EXPRESSION: WHO, WHERE AND WHEN?**

### 744 **A. Tissue expression and cellular sources of IL10**

745 In mammals it has been shown that IL10 can be produced by almost all leukocyte subtypes,  
746 with CD4<sup>+</sup> T cells and monocytes/macrophages being the most important sources.<sup>3</sup>  
747 Together with the identification of the sequence, the basal expression of *IL10* in different  
748 tissues has been reported for several non-mammalian vertebrates. Chicken and duck *IL10*  
749 showed higher expression in bursa and cecal tonsil and moderate expression in thymus,

750 liver and lung; no constitutive expression could be found in chicken spleen and bone  
751 marrow as well as in non-lymphoid tissues such as kidney, brain, heart and muscle. In  
752 contrast, in duck constitutive expression of *IL10* can be found in spleen and the highest  
753 expression is seen in lung.<sup>17,18</sup> In frogs, the highest constitutive expression is found in  
754 kidney, spleen and gut, and low expression is seen in liver or heart.<sup>19</sup> In teleost fish, the  
755 constitutive expression in different tissues varies among species.<sup>20–23,25–27,29,30</sup> Head kidney,  
756 gut and gills showed constitutive high expression in all investigated species; the same was  
757 true for spleen with the exception of fugu. The expression in isolated cell types was only  
758 determined in carp (Piazzon, manuscript in preparation) and goldfish,<sup>27</sup> where neutrophilic  
759 granulocytes and monocytes/macrophages are the cells expressing the highest levels of  
760 *IL10*. In rainbow trout the expression of the *IL10* paralogues was investigated in a  
761 mononuclear/macrophage-like cell line (RTS-11) showing that both paralogues can be  
762 expressed and are differentially regulated by various stimuli.<sup>31</sup>  
763 In chicken, bone-marrow derived macrophages and the HD11 macrophage cells line were  
764 shown to considerably upregulate *IL10* expression and protein production when stimulated  
765 with LPS.<sup>73</sup>  
766 In fish, other than immune cells, the epithelial cell line from fathead minnow (EPC) is able  
767 to express *IL10* and its expression is regulated by poly I:C and ranavirus infections.<sup>102</sup>  
768 Similarly, in rainbow trout, the epithelial cell line RTL from liver, the fibroid cell lines  
769 RTG-2 from gonad, and RTGill from gills, were all shown to express *IL10* and  
770 differentially regulate its expression upon poly I:C, LPS or IFN $\gamma$  stimulation.<sup>22</sup> It is  
771 important to note that observed differences between species can be due to the use of  
772 different techniques to measure expression, some used real time-quantitative PCR while  
773 others used standard PCR with lower detection limits. Other differences, such as the

774 expression in PBMC (PBL in fish), can be attributed to the different composition of  
775 circulating leukocytes that varies greatly among species. Despite this, we can state that in  
776 general, there is high expression of *IL10* transcripts in mucosal tissues such as gut, gills or  
777 lungs. This expression pattern is expected owing to the homeostatic and tolerogenic role  
778 played by IL10 at these surfaces, preventing excessive immune responses against ingested  
779 or inhaled antigens as well as microbiota at mucosal sites. Immune tissues such as spleen,  
780 avian bursa and cecal tonsil, or fish head kidney also generally present high constitutive  
781 expression of this cytokine.

782

### 783 **B. Kinetics of *IL10* expression**

784 IL10 expression is highly regulated and is generally expected following or concomitantly  
785 with the expression of pro-inflammatory mediators. Several studies show that *IL10* is  
786 upregulated by proinflammatory molecules such as bacterial and viral PAMPS. For  
787 instance, LPS, Poly I:C, bacterial and mitogen stimulations rapidly increase the expression  
788 of *IL10* on chicken, duck, frog and fish cells and tissues. The induction of the expression of  
789 this cytokine starts quite early; peaks between 6 and 24 h depending on the species, tissue  
790 and treatment, and goes down gradually generally lasting longer than the expression of the  
791 pro-inflammatory genes.<sup>17,19,21–23,25,26,29,73,103</sup> This early induction has been proposed as a  
792 “self-control” mechanism to limit collateral damage caused by exaggerated  
793 inflammation.<sup>103,104</sup> TNF $\alpha$  stimulation of goldfish monocytes and macrophages  
794 downregulated *IL10* expression corroborating the presence of the TNF $\alpha$  responsive element  
795 reported in fugu.<sup>20,27</sup>

796 IL10 can also be induced by anti-inflammatory mediators such as TGFβ1.<sup>30</sup> LPS-stimulated  
797 grass carp monocytes start producing proinflammatory mediators in the first hours and  
798 shortly after, the expression of *TGFβ1* and *IL10* starts and remains high controlling the  
799 expression of the pro-inflammatory mediators.<sup>77</sup>

800 To note, most of the aforementioned studies refer to gene expression data although it was  
801 already previously mentioned that IL10 is highly regulated also at posttranscriptional  
802 levels. Therefore, the development of specific antibodies for the analysis of protein levels  
803 would be crucial. This would allow to study the ratio of pro-/anti-inflammatory cytokines  
804 in various cell types upon treatment or infection and to obtain valuable information about  
805 the regulation of this cytokine at total protein levels.

806

### 807 **C. Role of IL10 in infection, inflammation and in stress**

808 The ability of certain cells or tissues to express *IL10* has been related to different  
809 pathogenic or stress situations. In some cases, differential expression of IL10 in different  
810 genetic backgrounds was associated to disease resistance or susceptibility. For instance, in  
811 chicken, susceptible and resistant animals have been described regarding *Eimeria maxima*  
812 (protozoan intestinal parasite) infections. Upon infection, susceptible birds show increased  
813 *IL10* expression in the small intestine when compared to resistant birds. Although the  
814 expression of other proinflammatory mediators such as IFNγ does not seem to be affected,  
815 the high levels of IL10 in the susceptible line may counteract inflammation, possibly  
816 contributing to the inability of these animals to limit the growth of the parasite. Uninfected  
817 animals already show an important difference in the constitutive expression of *IL10*, as

818 spleens of susceptible birds express 43 fold higher amounts of *IL10* when compared to the  
819 resistant line.<sup>18</sup>

820 A similar observation was obtained in teleost fish. Two different common carp strains have  
821 been described to present different susceptibilities and mortality rates upon infection with  
822 the haemoflagellate parasite *Trypanoplasma borreli*. While the resistant strain shows  
823 upregulation of *IL10* in later phases of the infection coinciding with the downregulation of  
824 proinflammatory genes and increase in specific antibodies, the susceptible line shows an  
825 abnormal early expression of *IL10* leading to a reduced inflammatory response and higher  
826 mortalities (Fig. 6).<sup>105</sup> Again, in common carp, a single nucleotide polymorphism in the  
827 *IL10a* promoter has been strongly associated to resistance against cyprinid herpes virus 3  
828 infections.<sup>32</sup>

829 It is clear that also in non-mammalian vertebrates a well-regulated expression of *IL10*  
830 during the course of the inflammatory processes is crucial, and dysregulation of the IL10  
831 network has been associated with mortalities or higher disease susceptibility. For example,  
832 specific chicken breeds with impaired *IL10* expression show prolonged inflammation and  
833 infectious symptoms when exposed to *Campylobacter jejuni*, a commensal bacteria in  
834 chicken.<sup>106</sup> It is widely accepted that correct IL10 regulation and expression is especially  
835 important in maintaining gut homeostasis, and dysregulation of this molecule leads to  
836 pathologic situations such as inflammatory bowel disease or ulcerative colitis widely  
837 studied in mammals.<sup>107</sup> In Tetraodon, ablation of regulatory T cells through administration  
838 of neutralizing anti-CD25 antibodies, led to a decrease in *IL10* expression in the gut and to  
839 an increase in pro-inflammatory gene expression as well as intestinal lesions.<sup>108</sup> The data  
840 presented in this study closely resemble those seen in mammalian models of gut  
841 inflammation, nevertheless, a direct link between IL10 levels and disease outcome needs to

842 be formally proven. Other studies in fish focusing on the enteritis model, tried to find a link  
843 between IL10 and intestinal health. In zebrafish, oxazolone-induced enterocolitis was  
844 characterized by an increased expression of *IL10* together with *IL1 $\beta$*  and *TNF $\alpha$* .<sup>109</sup> When  
845 common carp are fed with soy containing feeds they develop transient enteritis and recover  
846 after 4 weeks. During this process *IL10* upregulation was observed already after 1 week of  
847 feeding, more or less coinciding with the peak of inflammation and with the upregulation of  
848 *IL1 $\beta$* . The anti-inflammatory molecule that was upregulated during the recovery phase was  
849 *TGF $\beta$* .<sup>110</sup> In the case of the pathogenic enteritis caused by *Enteromyxum leei* in seabream,  
850 *IL10* showed the highest upregulation among all the interleukin genes studied in the gut and  
851 this upregulation is much prominent in later phases of infection<sup>111</sup> coinciding with the peak  
852 of antibody production (unpublished observation). The use of probiotics and  
853 immunostimulants in animal feed also showed to regulate IL10 levels. The introduction of  
854 *Saccharomyces boulardii* in chicken diets induced a higher IL10 production in gut and at  
855 the same time an increased number in IgA positive cells and positive effects on intestinal  
856 ultrastructure.<sup>112</sup> The upregulation of an immunosuppressive gene upon stimulatory  
857 conditions can be interpreted as a compensatory mechanism to regulate exaggerated  
858 responses that can be caused by the immunostimulant.

859 Altogether, when focusing on intestinal infections or inflammation models IL10 seems to  
860 have a conserved regulatory role in the gut of mammalian and non-mammalian species.  
861 More studies focusing on the kinetics of IL10 expression especially at protein level will be  
862 needed to better understand the multifaceted aspects of IL10 function and the role of IL10-  
863 producing leukocytes, particularly at mucosal surfaces.

864 Additional (indirect) evidence of the crucial role of IL10 in the regulation of immune  
865 responses to infections comes from several in vitro and in vivo studies mostly using  
866 intracellular pathogens, in which the pathogen itself has been shown to possibly manipulate  
867 or interfere with *IL10* expression. For instance, the facultative intracellular bacteria  
868 *Franciella noatunensis* can infect Atlantic cod macrophages inducing an elevated  
869 expression of *IL10*. This has been related to a downregulation of *IL1 $\beta$* , *IL6*, *IL8* and *IFN $\gamma$*   
870 which in turn has been proposed as a mechanism of the pathogen to regulate the host  
871 immune response.<sup>113,114</sup> Mammalian mycobacterial species have been shown to increase  
872 SOCS3 levels as a strategy to downregulate inflammation.<sup>115</sup> In *Mycobacterium marinum*-  
873 infected goldfish elevated expression of *IL10* and *SOCS3* has also been observed but  
874 whether upregulation of *SOCS3* is caused directly by the bacteria or by increased levels of  
875 IL10 is yet to be determined.<sup>116,117</sup> Upon infection with infectious pancreatic necrosis virus  
876 (IPNV) Atlantic salmon spleen, head kidney and liver increase the expression of *IL10* and  
877 this is also proposed as a pathogen strategy to control the inflammatory response induced  
878 by *IFN $\gamma$*  and favor a switch towards an anti-inflammatory state.<sup>118</sup> Actually, the strategy of  
879 regulating the cytokine network of the host, and more specifically, the use of anti-  
880 inflammatory molecules such as IL10, is well known for several pathogens, including  
881 viruses.<sup>82,83</sup> As seen before, some viruses encode their own IL10 homologs to regulate the  
882 host immune responses further highlighting the pivotal role of IL10 in protection against  
883 infections.

884 Finally, the expression of this cytokine has also been used as a marker for animal welfare as  
885 its expression has been linked to certain stressful conditions or to the presence of specific  
886 pollutants. It is known that tributyltin, a wide spread marine pollutant, cause

887 immunosuppressive effects in some fish species. This immunosuppressive effects have  
888 been linked to an increased expression of *IL10* and *TGFβ1* caused by this pollutant in  
889 Atlantic salmon.<sup>119</sup> An increase in serum IL10 was found in an experimental handling stress  
890 model in goldfish. In this study IL10 levels are proposed as an additional stress indicator  
891 together with cortisol and glucose levels.<sup>120</sup>  
892 Altogether, the data accumulated so far, mostly in chicken and several teleost fish species,  
893 point towards a strong conservation of the regulatory role of IL10 during infection and  
894 inflammation. Even in non-mammalian vertebrates, manipulation of the IL10 network has  
895 to be approached carefully as exaggerated expression of this cytokine can lead to an  
896 immunosuppressive state facilitating pathogen invasion, whereas impaired expression can  
897 lead to excessive inflammation and damage.

898

## 899 **VI. ZEBRAFISH MODEL AS A TOOL TO STUDY IL10**

900 Over the past years the zebrafish model has established itself as a great tool to study  
901 fundamental questions in developmental biology but most of all, it has recently emerged as  
902 a suitable tool to investigate biomedical questions also related to human diseases.<sup>121</sup>

903 Zebrafish is currently being used as a model to study genes involved in tumor  
904 progression,<sup>122</sup> stem cell development and differentiation,<sup>123</sup> several infection models of  
905 host-pathogen interaction,<sup>124,125</sup> drug discovery<sup>126</sup> as well as metabolic disease.<sup>127,128</sup>

906 Owing to the availability of an ever growing number of transgenic zebrafish lines  
907 expressing reporter fluorescent proteins under the control of several immune cell-specific  
908 promoters, there is no doubt that the zebrafish model will serve as an additional tool to help  
909 dissect IL10 biology in fish as well. Where antibodies are not available, the use of

910 transgenic reporter zebrafish lines expressing fluorescent proteins under the control of the  
911 IL10 promotor can help elucidate the source(s) of IL10 expression in immune and non-  
912 immune cells. Since cells can be imaged in vivo at various time points without sacrificing  
913 the animal or sorting the cells, the kinetics of IL10 expression can be concomitantly imaged  
914 in various cell types.

915 Most importantly, very recently, IL10 knockout mutants became available  
916 ([http://www.sanger.ac.uk/sanger/Zebrafish\\_Zmpgene/ENSDARG00000078147](http://www.sanger.ac.uk/sanger/Zebrafish_Zmpgene/ENSDARG00000078147)) and can  
917 help to further elucidate the role of IL10 during infection and diseases. For example, the  
918 possibility to image in real-time the kinetics of cell recruitment during tumor progression or  
919 host-pathogen interaction during infections in an IL10 transgenic or in an IL10 knockout  
920 background, will allow for a complementation and refinement of the approaches used to  
921 date to investigate IL10 functions. Finally, through the use of knockdown or knockout  
922 approaches for the candidates of the IL10 receptor complex, in a manner similar to the one  
923 used for the discovery of the type I IFN receptor complex,<sup>129</sup> it will be possible to  
924 unequivocally ascertain the role of CRFB4 in the formation of the fish IL10 receptor  
925 complex with CRFB7. Altogether, we think that the zebrafish model will provide numerous  
926 possibilities to expand, complement and validate the study of this (and other cytokines), not  
927 only in fish, but in all vertebrate species.

928

## 929 **CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

930 In this review we have shown that from the data accumulated thus far, there is strong  
931 evidence suggesting that the structure, intracellular signaling, and overall biological  
932 functions of IL10 are strongly conserved throughout vertebrate evolution. The functional  
933 studies, performed mainly in chicken and teleost fish, point towards a conservation of the

934 anti-inflammatory activities of IL10 on phagocytes and to a crucial regulatory role of IL10  
935 in gut homeostasis. Knowledge on the role of IL10 on lymphocytes is still scarce and only  
936 addressed in fish; the association of IL10 with disease susceptibility or resistance has also  
937 been partly addressed, again confirming the pivotal role of this cytokine in the regulation of  
938 exacerbated inflammatory responses also in non-mammalian vertebrates. Finally, the  
939 identification and functional characterization of the first non-mammalian viral IL10  
940 homologue in a cyprinid herpesvirus, *CyHV3*IL10, supports the various observations  
941 suggesting that manipulation of the IL10 pathway can be sufficient to tip the balance  
942 between disease susceptibility and resistance.

943 Despite the many advances made in the last years in the understanding of IL10 biology and  
944 function in non-mammalian vertebrates, still a few pieces of the puzzle remain open. For  
945 example, the presence in some teleost species of duplicated genes, for both ligands and  
946 receptors, together with the observation that gene duplication does not necessarily imply  
947 functional redundancy (Piazzon, manuscript in preparation),<sup>31</sup> certainly increases the level  
948 of complexity of IL10 regulation in Teleosts and the role of each of the paralogues still  
949 needs to be investigated in detail. Progress, although substantial, has been greatly slowed  
950 down by the lack of tools (recombinant proteins, antibodies) in most non-mammalian  
951 vertebrates. Nevertheless, cross-reactive inhibitors or antibodies can be found, especially  
952 against transcription factors. The latter are usually well conserved molecules, and it has  
953 been relatively easy to find cross-reactive antibodies, as for example against STAT3 and  
954 phosphorylated STAT3.<sup>27,61</sup> Nowadays, the genomes and transcriptomes of hundreds of  
955 species are available in the databases. This increased enormously the possibilities to  
956 perform *in silico* analyses and comparative studies in almost any vertebrate class.  
957 Regardless, it is important to be aware that most of the molecules found in these databases

958 are automatic predictions and their automatic annotation should always be supported by  
959 functional analysis. Finally, functional data on the biological activities of IL10 in reptiles,  
960 amphibians and modern bony fish (coelacanth or lungfish) are completely lacking and  
961 would certainly add important pieces to the evolutionary puzzle of IL10 evolution.

962

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970

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1316

1317 **FIG. 1. The IL10 protein is present and conserved in all vertebrate species.**

1318 Phylogenetic tree analysis of full length IL10 protein sequences from selected species  
1319 within each relevant group. The tree was constructed using the neighbor joining method  
1320 within the MEGA 6 package and bootstrapped 10000 times. Bootstrap values over 50% are  
1321 shown. Duplicated genes in rainbow trout (*Oncorhynchus mykiss*) and common carp  
1322 (*Cyprinus carpio*) are indicated as (a) and (b) next to the species name. IL10 protein  
1323 sequences can be found in all vertebrate species and it groups within each class of  
1324 vertebrate. The low bootstrap values obtained are due to high sequence similarity but the  
1325 tree is supported by the perfect grouping of each class of vertebrate. The accession numbers  
1326 of the sequences used to perform the analysis are the following: NP\_000563 *Homo sapiens*,  
1327 NP\_036986 *Rattus norvegicus*, NP\_034678 *Mus musculus*, NP\_999206 *Sus scrofa*,  
1328 NP\_776513 *Bos taurus*, NP\_001003077 *Canis lupus familiaris*, XP\_006922887 *Pteropus*  
1329 *alecto*, XP\_006754445 *Myotis davidii*, NP\_001075514 *Oryctolagus cuniculus*, ALG04628  
1330 *Lepus europaeus*, XP\_007523171 *Erinaceus europaeus*, XP\_004610114 *Sorex araneus*,  
1331 ELW47753 *Tupaia chinensis*, ABQ40392 *Dasypus novemcinctus*, XP\_003410325  
1332 *Loxodonta africana*, AIA08972 *Elephas maximus*, AAD01799 *Trichosurus vulpecula*,  
1333 AFY22677 *Phascolarctos cinereus*, XP\_007668455 *Ornithorhynchus anatinus*,  
1334 XP\_010402880 *Corvus cornix cornix*, XP\_014728054 *Sturnus vulgaris*, XP\_010304693  
1335 *Balearica regulorum gibbericeps*, XP\_010158678 *Eurypyga helias*, XP\_009646203  
1336 *Egretta garzetta*, XP\_009463847 *Nipponia nippon*, NP\_001004414 *Gallus gallus*,  
1337 BAL02992 *Coturnix japonica*, NP\_001297297 *Anas platyrhynchos*, XP\_013045032 *Anser*  
1338 *cygnoides domesticus*, XP\_005230381 *Falco peregrinus*, XP\_011591578 *Aquila*  
1339 *chrysaetos canadensis*, XP\_009325615 *Pygoscelis adeliae*, XP\_009271033 *Aptenodytes*  
1340 *forsteri*, KQL51993 *Amazona aestiva*, XP\_005143250 *Melopsittacus undulatus*,

1341 XP\_009956868 *Leptosomus discolor*, XP\_008936084 *Merops nubicus*, XP\_014803968  
1342 *Calidris pugnax*, XP\_009886505 *Charadrius vociferus*, XP\_010086506 *Pterocles*  
1343 *gutturalis*, XP\_009581167 *Fulmarus glacialis*, EMC81973 *Columba livia*, XP\_009895472  
1344 *Picoides pubescens*, XP\_009995817 *Chaetura pelagica*, XP\_009940291 *Opisthocomus*  
1345 *hoazin*, XP\_009562150 *Cuculus canorus*, XP\_010165026 *Caprimulgus carolinensis*,  
1346 XP\_008498919 *Calypte anna*, ADU34193 *Carassius auratus*, AAW78362 *Danio rerio*,  
1347 cypCar\_00007086 *Cyprinus carpio*(a), cypCar\_00012555 *Cyprinus carpio*(b),  
1348 XP\_015227932 *Cyprinodon variegatus*, XP\_014868952 *Poecilia mexicana*, BAD20648  
1349 *Oncorhynchus mykiss*(a), FR691804 *Oncorhynchus mykiss*(b), ABM46995 *Salmo salar*,  
1350 XP\_004545126 *Maylandia zebra*, XP\_005924770 *Haplochromis burtoni*, AAP57415  
1351 *Tetraodon nigroviridis*, CAD62446 *Takifugu rubripes*, KKF31567 *Larimichthys crocea*,  
1352 XP\_006628630 *Lepisosteus oculatus*, AHX22596 *Paralichthys olivaceus*, XP\_008318394  
1353 *Cynoglossus semilaevis*, XP\_010872914 *Esox lucius*, XP\_010786179 *Notothenia coriiceps*,  
1354 AJO68021 *Epinephelus coioides*, XP\_004069312 *Oryzias latipes*, AJA39866 *Seriola*  
1355 *dumerili*, XP\_007247805 *Astyanax mexicanus*, XP\_008294254 *Stegastes partitus*,  
1356 CAK29522 *Dicentrarchus labrax*, AGS55345 *Sparus aurata*, ABV64720 *Gadus morhua*,  
1357 XP\_013911813 *Thamnophis sirtalis*, XP\_007437603 *Python bivittatus*, XP\_003224060  
1358 *Anolis carolinensis*, XP\_015283261 *Gekko japonicus*, EMP30816 *Chelonia mydas*,  
1359 XP\_005306530 *Chrysemys picta bellii*, XP\_006267889 *Alligator mississippiensis*,  
1360 XP\_006024846 *Alligator sinensis*, CAE92388 *Xenopus laevis*, NP\_001165400 *Xenopus*  
1361 *tropicalis*, XP\_007897740 *Callorhinchus milii*, XP\_006000454 *Latimeria chalumnae*.  
1362  
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1364

1365 **FIG. 2. Genomic organization of the *IL10* locus, the *IL10* gene structure and the IL10**  
1366 **protein are conserved across vertebrates. A)** Schematic organization of the *IL10* locus  
1367 using the gene orders on the human chromosome as reference. The information of the gene  
1368 order was retrieved from ensemble (<http://www.ensembl.org/>) using the following genome  
1369 assemblies: Human GRCh38.p5, Mouse GRCm38.p4, Chicken Galgal4, Chinese softshell  
1370 turtle PelSin\_1.0, Frog JGI4.2, Coelacanth LatCha1, Fugu FUGU4.0 and Zebrafish  
1371 GRCz10. The syntenic conserved orthologs or gene blocks are shown in matching colors.  
1372 Asterisks (\*) next to the chromosome (Ch) or scaffold (scaf) name indicates that the  
1373 orientation was inverted to optimize the alignment. Note the overall syntenic conservation  
1374 of the *IL10* locus across vertebrate species. **B)** Intron/Exon organization and length of the  
1375 *IL10* gene in various vertebrate species in which the *IL10* sequence was characterized in  
1376 detail. The schematics were constructed using <http://wormweb.org/exonintron>. When the  
1377 gene is present in duplicate copy, the paralogues are indicated as (a) or (b) next to the  
1378 species name. The numbers on the introns of the human gene denote the phase of the intron.  
1379 Red vertical lines indicate the sites and number of instability motifs (ATTTA) in the  
1380 untranslated regions. Note the conservation of the gene structure with 5 exons and 4  
1381 introns. While exons retained the same length, intron size varied greatly among species. In  
1382 amphibians, introns are a little longer than in human<sup>19</sup> while birds present shorter introns  
1383 when compared to mammals.<sup>18</sup> The bird *IL10* gene is still 1.5-2 times longer than the fish  
1384 *IL10*, making the fish gene the most compact one,<sup>21,23,25</sup> with the exception of trout<sup>22</sup> that  
1385 presents introns of similar size to the mammalian counterparts. **C)** Amino acid sequence  
1386 alignment of IL10 from several species (accession numbers in Fig.1) performed with  
1387 PROMALS3D (<http://prodata.swmed.edu/promals3d>) using the crystal structure of human  
1388 IL10 (PDB ID: 2H24) as a reference. Conserved cysteine residues are marked in black and

1389 the IL10R1 binding sites are indicated by the squares. The 27 residues predicted to make  
1390 contact with the human IL10R1 are color coded as follows: blue (complete conservation),  
1391 green (1-2 differences), yellow (3 differences), pink (50% conservation) and red (low  
1392 conservation). Consensus amino acid (aa) symbols at the bottom of the alignment are:  
1393 highly conserved aa are in bold and uppercase letters; aliphatic: *l*; aromatic: *@*;  
1394 hydrophobic: *h*; alcohol: *o*; polar residues: *p*; tiny: *t*; small: *s*; bulky residues: *b*; positively  
1395 charged: *+*; negatively charged: *-*; charged: *c*. Consensus secondary structure symbol “h”  
1396 points to the position of the conserved alpha helices. Numbers above the alignment indicate  
1397 the most conserved amino acids as compared to the human sequence, with 9 being the  
1398 highest conservation score as calculated by the AL2CO sequence conservation analysis  
1399 server <http://prodata.swmed.edu/al2co/al2co.php>. **D)** The sequences included in **C** were  
1400 modeled with Swiss-Model (<http://swissmodel.expasy.org/>) and all automatically fitted the  
1401 structure of human IL10 with good quality scores. The PDB files obtained were  
1402 manipulated with Jmol 14.6.0 to obtain the 3D representations of the IL10 homodimers  
1403 colored by domain. It is easily appreciated that all 3D structures are very similar and only  
1404 slight differences can be observed.

1405

1406 **FIG. 3. The IL10 receptor complex in mammalian and non-mammalian vertebrates.**

1407 **A) Schematic representation of the prototypical IL10R complex in mammals:** one IL10  
1408 molecule binds to two molecules of the IL10R1 which in turn recruits two molecules of the  
1409 IL10R2. This leads to the activation of the JAK1 and TYK2 kinases present in the  
1410 cytoplasmic tails of the receptors and subsequent phosphorylation of the tyrosine (Y)  
1411 residues at specific sites in the cytoplasmic tail of the IL10R1. Phosphorylated tyrosines  
1412 represent the docking sites for cytosolic STAT3, which in turn will be phosphorylated and

1413 will translocate to the nucleus. The binding site for JAK1 (PxxL) is highly conserved in all  
1414 investigated species, similarly to the TYK2 binding site, whereas the number of potential  
1415 phosphorylation sites in the IL10R1 tail varies among mammals and also between  
1416 mammals, birds and amphibians, indicated by dashed arrows (see further details in Fig.  
1417 4C). Although not investigated in detail at the functional level, orthologues of the IL10R  
1418 complex in amphibians can be identified based on conserved synteny (see also Fig. 4 and  
1419 5). In reptiles a conserved IL10R2 and an incomplete IL10R1 can be found at conserved  
1420 genomic locations. Therefore it is likely that the IL10R complex also in reptiles would have  
1421 a conserved structure although it still needs to be formally proven. **B) Schematic**  
1422 **representation of the putative IL10R complex in teleost fish:** identification of the  
1423 orthologues of the IL10R complex in teleost fish has proven more challenging due to the  
1424 lack of sequence conservation and weak preservation of genomic (synteny) structure.  
1425 Nevertheless, based on structural features and *in vitro* functional studies,<sup>47</sup> class II cytokine  
1426 receptor family-7 (CRFB7) has been without doubt annotated as IL10R1. Annotation of the  
1427 IL10R2 has proven more difficult due to the presence of two paralogues, CRFB4 and  
1428 CRFB5, which most likely are derived from a recent tandem duplication. Functional work  
1429 in grass carp<sup>69</sup> however, indicates that CRFB4 is most likely the co-receptor of the IL10R  
1430 complex in teleost whereas work in zebrafish supports the idea that CRFB5 is rather  
1431 involved in the type I IFNR complex (not shown). **C) Duplicated copies of all members**  
1432 **of the IL10R complex in some teleost fish:** in common carp, Atlantic salmon (and most  
1433 likely rainbow trout), duplicate copies of all members of the IL10R complex can be found.  
1434 This adds an extremely higher degree of complexity when considering all possible ligand-  
1435 receptor combinations. In the scheme a hypothetical complex has been depicted, but to date  
1436 it cannot be excluded that all combinations of subunits are possible.

1437 **FIG. 4. Genomic organization of the *IL10R1* locus and the *IL10R1* gene structure are**  
1438 **conserved across vertebrates. A)** Schematic organization of the *IL10R1* locus using the  
1439 gene orders on the human chromosome as reference. The information of the gene order was  
1440 retrieved from ensembl (<http://www.ensembl.org/>) using the following genome assemblies:  
1441 Human GRCh38.p5, Mouse GRCm38.p4, Chicken Galgal4, Green anole AnoCar2.0, Frog  
1442 JGI4.2, Fugu FUGU4.0 and Zebrafish GRCz10. The syntenic conserved orthologs or gene  
1443 blocks are shown in matching colors. Asterisks (\*) next to the chromosome (Ch) or scaffold  
1444 (scaf) name indicate that the orientation was inverted to optimize the alignment. **B)**  
1445 Intron/Exon organization and length of the *IL10R1* gene in various vertebrate species. The  
1446 Green anole sequence (as well as the one of other reptile *IL10R1*) is still incomplete in the  
1447 database and was not included. The schematics were constructed using  
1448 <http://wormweb.org/exonintron> and the intron/exon length information was retrieved from  
1449 ensembl. The numbers on the introns of the human gene denote the phase of the intron,  
1450 which is conserved in all species (not shown). Note the conservation of the gene structure  
1451 with 7 exons and 6 introns. While exons retained the same length, intron size varied among  
1452 species with chicken and fugu in particular, being the most compacted. Common carp  
1453 expresses two copies of the CRFB7 gene, but the genome assembly is still incomplete to  
1454 provide synteny information on the position of these genes in the carp genome. **C)** Amino  
1455 acid alignment of the cytoplasmic tails of various IL10R1/CRFB7 sequences in vertebrates.  
1456 Green highlights at the beginning indicate transmembrane regions; in light blue is the very  
1457 conserved JAK1 binding site (PxxL). In yellow are the two canonical STAT3-binding sites  
1458 (GYXXQ) found in all species, including the two non-canonical sites in frog (DYLLQ) and  
1459 in most fish species (GYRSG). Tetraodon is an exception to all species as it presents two  
1460 non-canonical sites (dark green) with substantially diverged sequences but at conserved

1461 positions with respect to the ones found in other vertebrates. In grey are the additional  
1462 STAT3-binding sites found upstream of the canonical ones in some mammalian and avian  
1463 sequences. In almost all sequences, an additional tyrosine (Y) residue is found downstream  
1464 of the canonical STAT3-binding sites (light grey), the function of which is still unknown.

1465

1466 **FIG. 5. The *IL10R2* locus and gene structure are not that well conserved among**

1467 **mammals and become complicated in teleost. A)** Schematic organization of the *IL10R2*

1468 locus using the gene orders on the human chromosome as reference. The information of the

1469 gene order was retrieved from ensembl (<http://www.ensembl.org/>) using the following

1470 genome assemblies: Human GRCh38.p5, Mouse GRCm38.p4, Chicken Galgal4, Chinese

1471 softshell turtle PelSin\_1.0, Frog JGI4.2, Stickleback BROAD S1, Tetraodon

1472 TETRAODON 8.0, Zebrafish GRCz10 and Atlantic salmon.<sup>72</sup> The syntenical conserved

1473 orthologs or gene blocks are shown in matching colors. Asterisks (\*) next to the

1474 chromosome (Ch) or scaffold (scaf) name indicate that the orientation was inverted to

1475 optimize the alignment. Teleost fish express two homologues of the mammalian IFNAR2

1476 gene, named CRFB1 and CRFB2, thus the color gradient in the IFNAR2 block; CRFB6 is

1477 homologous to mammalian IFNGR2 and, as discussed in the text, CRFB5 seems to act as

1478 the IFNAR1 functional equivalent while CRFB4 functions as IL10R2. Note the conserved

1479 synteny of the IFNAR2, IL10R2 and IFNAR1 gene cluster between mammals and birds,

1480 reptiles and amphibians. Such conservation is completely lost in and among fish genomes,

1481 making it more difficult to identify functional equivalent solely based on genome

1482 organization. Atlantic salmon presents multiple copies of several CRFB in this gene cluster,

1483 and similar to the Tetraodon expresses a CRFB3 gene not present in other fish species. **B)**

1484 Intron/Exon organization and length of the *IL10R2* gene in various vertebrate species. The

1485 schematics were constructed using <http://wormweb.org/exonintron> and the intron/exon  
1486 length information was retrieved from ensembl. The numbers on the introns of the human  
1487 gene denote the phase of the intron, which is conserved in all species (not shown). Note the  
1488 conservation of the gene structure with 7 exons and 6 introns. While exons retained the  
1489 same length, intron size varied among species.

1490

1491 **FIG. 6. Kinetics of IL10 expression in resistant and susceptible carp lines during a**  
1492 ***Trypanoplasma borreli* infection.** In resistant strains, the peak of pro-inflammatory  
1493 molecules expression (*iNOS*, *IFN $\gamma$*  and *TNF $\alpha$* ) closely follows the increase in parasitaemia  
1494 (black line). Upregulation of *IL10* (blue line) occurs in a later phase of the infection,  
1495 followed by a downregulation of pro-inflammatory genes, an increase in specific  
1496 antibodies, and ultimately by a reduced parasite burden. In contrast, in susceptible lines, an  
1497 early expression of *IL10* is observed, prior to a very modest upregulation of pro-  
1498 inflammatory genes. This leads to an uncontrolled parasite replication and increased  
1499 mortalities; (Modified from Forlenza et al 2011<sup>105</sup> and unpublished data from our group).

FIG 1:

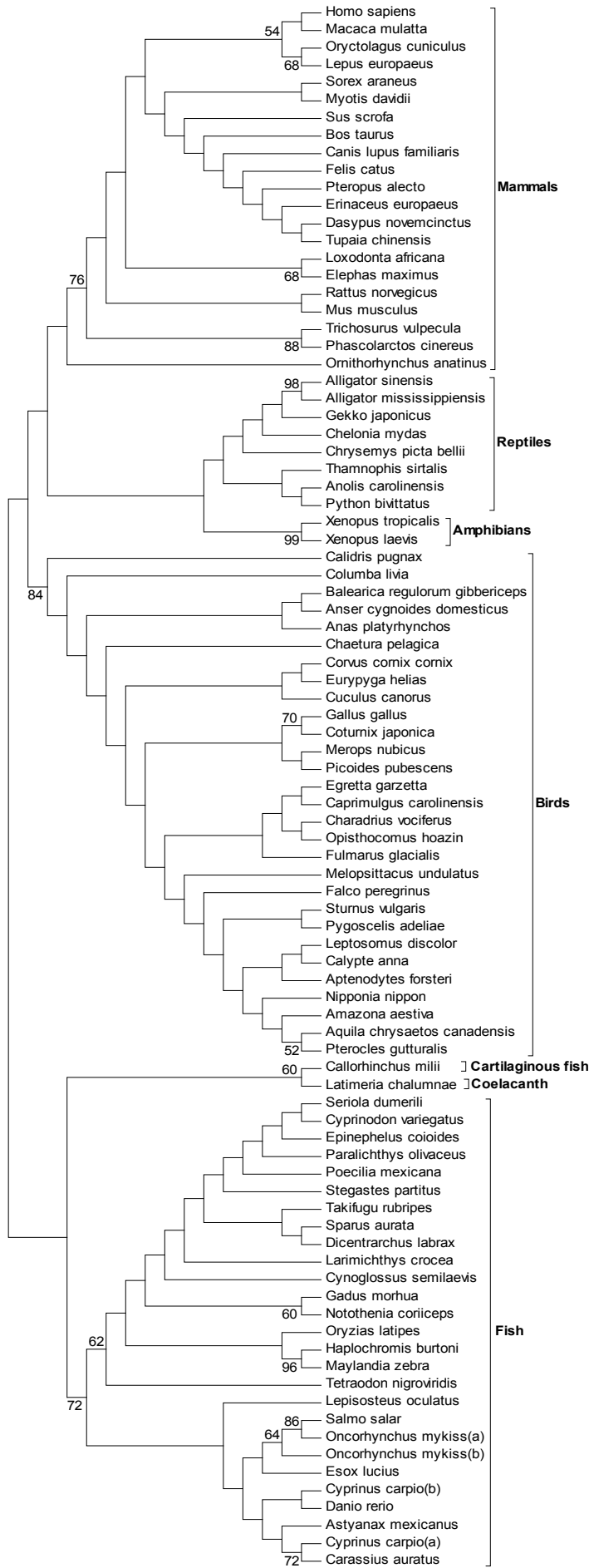




FIG 3:

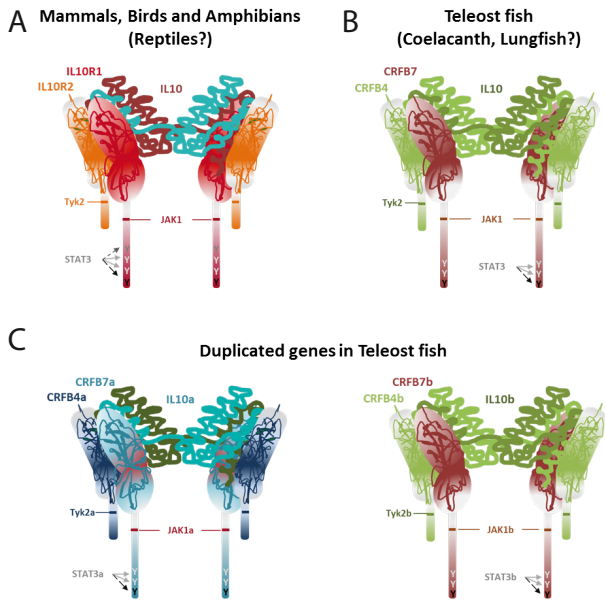
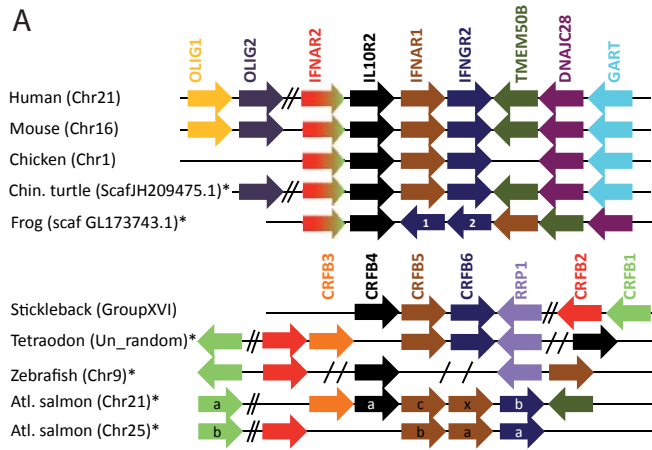




FIG 5:

A



B

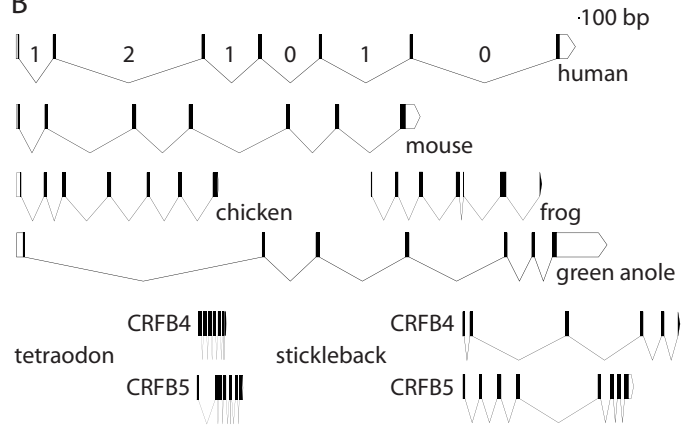


FIG 6:

