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**

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

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15

Abstract

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

I. INTRODUCTION

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

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

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

5

94   the immune system. It also directly inhibits proliferation of CD4$^+$ T cells,[8] IL2 and IFNγ

95   synthesis by Th1 cells and IL4 and IL5 synthesis by Th2 cells.[9,10] 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;[11,12] IL10 can also

100 stimulate NK cell proliferation and cytotoxic activity[13] as well as proliferation of specific

101 subsets of CD8$^+$ T cells.[14] 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.[15] IL10R1 is specific and has high affinity for IL10 while IL10R2 can

112 also act as co-receptor for other cytokines.[7] 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.[16]

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.[3,4,7] 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,[17]

145 chicken,[18] frog[19] and several teleost fish species (fugu,[20] common carp,[21] rainbow trout,[22]

146 zebrafish,[23,24] sea bass,[25] Atlantic cod,[26] goldfish,[27] Indian major carp[28,29] and grass carp[30])

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)[31,32] 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.[25] 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 polyadenilation signal (Fig. 2B). The instability motifs are rapid RNA

170 recognition sites for RNase E activity[33] important for post-transcriptional regulation of

171 genes. In chicken[18] and duck[17] *IL10* transcripts, 11 and 6 AUUUA motifs can be observed

172 in the 3'UTR; trout (a)[22] and grass carp[30] *IL10* transcripts present none, whereas sea bass,[25]

173 common carp (a and b)[21] and Indian major carp[29] *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.[27] 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[34]

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[35,36] 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),[31]

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. IFNγ stimulation specifically affects *IL10b* expression whereas bacterial

211   infections induce differential regulation of both paralogues depending on the tissue

212   studied.[31]

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[32] 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 4th 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

11

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.[37] Regarding *IL10* very few reports exist to that respect. A

244   new *IL10* splice variant lacking the entire exon 3, named IL10δ3, was described in human

245   leukemic cells and was associated with improved response to chemotherapy.[38] 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.[39] 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.[40,41]

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.[17] 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.[17] 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.[3] 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).[17,18,26]

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.[42,43] 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.[44]

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.[17,26]

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.[29,20] 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.[29] 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.[20] 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.[45]

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.[46] 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).[17–19,22,23,25] 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.[47] 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;[19] in sea bass the CD loop is longer than in humans and

339 helix E is smaller;[25] Indian major carp IL10 has helices A and F of different length.[28] 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.[17–23,25–31,48]

345 The residue I69 of human IL10, key for IL10 immunostimulatory functions[49] 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.[18–20,23,27,30] Trout and sea bass IL10 have one

348 potential N-glycosylation site[22,25], fugu has two[20] and chicken and zebrafish IL10[18,23] 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.[49,50]

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.[28] 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).[51,52] 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.[53] The latter activates the Janus kinases Jak1 and Tyk2 associated with the

370   cytoplasmic tails of IL10R1 and IL10R2 respectively.[54,55] All this leads to phosphorylation

371   of STAT3 or other latent transcription factors depending on the cell type.[56,57]

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).[58,59] 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[52], zebrafish[47], and goldfish;[47] 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*, *FXYD6* 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.[47] 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.[47,52] 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).[47,52] 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[52] 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,[47]

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,[60] 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)

19

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.[4] 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.[47,52,61] 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.[47,61] 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.[47] 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.[52] 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.[47]

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.[62] 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.[63–65] In mammals, the gene cluster *IFN-α receptor-2* (*IFNAR2*),

20

445   *IL10R2, IFNAR1* is a very conserved group of synteny.[66] 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).[51] 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.[58] 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);[67] 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)[68] 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)[59] 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.[69]

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.[59] 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 *IFNAR1* 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.[58,69–71] The expression levels remain stable in most cells even after

500   activation.[65,71] 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 β-strands, but chicken and duck proteins only present 3 of these 4 conserved

505   residues.[70]

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[72] 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.[3] 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.[27,30,61] 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.[27] *SOCS3*

531 expression, in fish as in mammals, is also upregulated within the first hours of exposure to

532 IL10[27,30,61] and this effect can be abolished by a STAT3 inhibitor.[30] 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).[3] 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.[3,6]

563   The only study in chicken addressing the inhibitory activity of IL10 on macrophages made

564   use of neutralizing antibodies against chicken IL10.[73] 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 IFNγ stimulation as well as the expression of several pro-inflammatory

574   genes including *TNFα1*, *TNFα2*, *IL10*, *CXCL8* and the NADPH oxidase component

575   $p47^{phox}$. Under the same conditions, goldfish splenocytes showed downregulation of the

576   expression of *IFNγ*.[27] 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   TNFα rather than directly on radical production and release.[74,75] In the case of goldfish,

579   besides downregulation of *TNFα1* and *TNFα2*, a direct effect of IL10 on the respiratory

580   burst was demonstrated due to the direct downregulation of NADPH oxidase components.[27]

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.[61,76] 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, *IL1β, TNFα, 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[61] and the surface expression of

590   MHCI protein.[76] 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 *TNFα, IL1β, IL8* and *iNOS* in

604   monocytes/macrophages.[77] On the same cells the authors also tested the effect that

605   endogenous IL10 had on TGFβ1 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 TGFβ1 mRNA and

608   protein levels increased. When IL10 and TGFβ1 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 TGFβ1 on LPS-induced NFκB activation was also

612   investigated. The protein IκBα, which inhibits NFκB by blocking its ability to bind DNA,

613   is degraded in grass carp monocytes/macrophages upon LPS stimulation. Both, IL10 and

614   TGFβ1 showed the ability to block LPS-induced IκBα protein degradation thereby

615   attenuating the pro-inflammatory effect of LPS.[77]

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.[78] 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[11,12] and to promote proliferation of subsets of $CD8^+$ T lymphocytes.[14] On the

629   other hand, it directly inhibits cytokine synthesis and proliferation of $CD4^+$ Th1 and Th2

630   lymphocytes, indirectly affecting the progression or the resolution of the adaptive immune

631   responses.[8–10] 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 IFNγ 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 IFNγ, indirectly affecting the activity of the phagocytes.[18] Duck

643   recombinant IL10 inhibits the expression of IL2 induced by mitogen stimulation of

644   PBMCs.[17]

645   In teleost fish, recombinant carp IL10 inhibited the IL2-induced proliferation of

646   thymocytes.[76] This is in contrast with the activity of mammalian IL10 on the same cell

647   type[79] 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.[61] 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

29

655   of CD8^{+} 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[80] allowed for the study of the effect of

659   IL10 specifically on IgM^{+} B cells. Recombinant carp IL10 directly promoted IgM[+] 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^{+} B cells.[61,76] Contrary to what was found in neutrophils, IL10

663   increased the surface expression of MHCI molecules in IgM^{+} B cells possibly improving

664   antigen presentation by these cells.[76] 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.[61] These studies in carp show well

670   conserved bioactivity of IL10 on B cells when compared to mammals but focus only on

671   IgM^{+} B cells. To complete these studies, the effect of fish IL10 on IgT[+] and IgD^{+} 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.[30]

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.[81] 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.[82,83] Among the most studied

692   IL10 viral homologs are those produced by the human Cytomegalovirus (CMV)[84] and

693   Epstein-Barr virus (EBV),[2] although more than 20 cytokine homologs have been described

694   in viruses infecting mammals including horse,[85] monkeys,[86] sheep,[87,88] cow,[89] goat,[90]

695   camel[91] and even bats.[92] This phenomenon is not restricted to mammals, as several viruses

696   infecting birds (pigeon pox virus, penguin pox virus[93] and canary pox virus),[94] reptiles

697   (testudinid herpesvirus)[95] and fish (anguillid herpesvirus 1[96] and cyprinid herpesvirus 3)[97]

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 $_{CMV}$IL10 and $_{EBV}$IL10, functional studies on the biological

702 activities of viral cytokine homologues have been conducted only on the cyprinid

703 herpesvirus 3 IL10 homologue ($_{CyHV3}$IL10).

704 Open Reading Frame 134 (ORF134) of CyHV3 encodes for the $_{CyHV3}$IL10, which was

705 shown to be the second most abundant protein in the virus secretome.[98] 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.[99] The predicted

708 three-dimensional structure and residues important for the interaction with the IL10R1 are

709 highly conserved.[48] Indirect evidence of $_{CyHV3}$IL10 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 $_{CyHV3}$IL10 and zebrafish IL10.[99] More direct evidence was

712 provided by work in common carp, in which recombinant $_{CyHV3}$IL10 was shown to induce

713 phosphorylation of STAT3 and expression of *SOCS3* in carp leukocytes.[76] Furthermore,

714 recombinant $_{CyHV3}$IL10 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$^+$ B cells and of

717 certain subsets of memory CD8$^+$ T cells.[76] In zebrafish, injections of $_{CyHV3}$IL10 mRNA

718 induced an increase in the number of lysozyme-positive cells in zebrafish embryos in a

719 manner similar to zebrafish IL10.[99] Nevertheless, similarly to some mammalian viral

720 cytokines such as $_{EBV}$IL10, it does not mimic the full repertoire of host IL10 activities.

721 $_{CyHV3}$IL10 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.[76]

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[100] and inhibition of apoptosis,[101] 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.[98] 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$^+$ T cells and monocytes/macrophages being the most important sources.[3]

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.[17,18] In frogs, the highest constitutive expression is found in

754 kidney, spleen and gut, and low expression is seen in liver or heart.[19] In teleost fish, the

755 constitutive expression in different tissues varies among species.[20–23,25–27,29,30] 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,[27] 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.[31]

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.[73]

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.[102]

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 IFNγ stimulation.[22] 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. [17,19,21–23,25,26,29,73,103] This early induction has been proposed as a

792 "self-control" mechanism to limit collateral damage caused by exaggerated

793 inflammation.[103,104] TNFα stimulation of goldfish monocytes and macrophages

794 downregulated *IL10* expression corroborating the presence of the TNFα responsive element

795 reported in fugu.[20,27]

796 IL10 can also be induced by anti-inflammatory mediators such as TGFβ1.[30] 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.[77]

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.[18]

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).[105] 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.[32]

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.[106] 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.[107] 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.[108] 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 *IL1β* and *TNFα*.[109] 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   *IL1β*. The anti-inflammatory molecule that was upregulated during the recovery phase was

849   *TGFβ*.[110] 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[111] 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.[112] 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 *IL1β, IL6, IL8* and *IFNγ*

870 which in turn has been proposed as a mechanism of the pathogen to regulate the host

871 immune response.[113,114] Mammalian mycobacterial species have been shown to increase

872 SOCS3 levels as a strategy to downregulate inflammation.[115] 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.[116,117] 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 IFNγ and favor a switch towards an anti-inflammatory state.[118] 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.[82,83] 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 tributylin, 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.[119] 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.[120]

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 immunosuppresive 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.[121]

903 Zebrafish is currently being used as a model to study genes involved in tumor

904 progression,[122] stem cell development and differentiation,[123] several infection models of

905 host-pathogen interaction,[124,125] drug discovery[126] as well as metabolic disease.[127,128]

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 promotors, 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) 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,[129] 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),[31] 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.[27,61] 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

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 boostrap 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.*

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FIG. 2. Genomic organization of the *IL10* locus, the *IL10* gene structure and the IL10 protein are conserved across vertebrates. **A)** Schematic organization of the *IL10* locus using the gene orders on the human chromosome as reference. The information of the gene order was retrieved from ensemble (http://www.ensembl.org/) using the following genome assemblies: Human GRCh38.p5, Mouse GRCm38.p4, Chicken Galgal4, Chinese softshell turtle PelSin_1.0, Frog JGI4.2, Coelacanth LatCha1, Fugu FUGU4.0 and Zebrafish GRCz10. The syntenic conserved orthologs or gene blocks are shown in matching colors. Asterisks (*) next to the chromosome (Ch) or scaffold (scaf) name indicates that the orientation was inverted to optimize the alignment. Note the overall syntenic conservation of the *IL10* locus across vertebrate species. **B)** Intron/Exon organization and length of the *IL10* gene in various vertebrate species in which the *IL10* sequence was characterized in detail. The schematics were constructed using http://wormweb.org/exonintron. When the gene is present in duplicate copy, the paralogues are indicated as (a) or (b) next to the species name. The numbers on the introns of the human gene denote the phase of the intron. Red vertical lines indicate the sites and number of instability motifs (ATTTA) in the untranslated regions. Note the conservation of the gene structure with 5 exons and 4 introns. While exons retained the same length, intron size varied greatly among species. In amphibians, introns are a little longer than in human[19] while birds present shorter introns when compared to mammals.[18] The bird *IL10* gene is still 1.5-2 times longer than the fish *IL10*, making the fish gene the most compact one,[21,23,25] with the exception of trout[22] that presents introns of similar size to the mammalian counterparts. **C)** Amino acid sequence alignment of IL10 from several species (accession numbers in Fig.1) performed with PROMALS3D (http://prodata.swmed.edu/promals3d) using the crystal structure of human 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 sited 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,[47] 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[69] 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

positions with respect to the ones found in other vertebrates. In grey are the additional

STAT3-binding sites found upstream of the canonical ones in some mammalian and avian

sequences. In almost all sequences, an additional tyrosine (Y) residue is found downstream

of the canonical STAT3-binding sites (light grey), the function of which is still unknown.

FIG. 5. The *IL10R2* locus and gene structure are not that well conserved among
mammals and become complicated in teleost. A) Schematic organization of the *IL10R2*

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

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

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

softshell turtle PelSin_1.0, Frog JGI4.2, Stickleback BROAD S1, Tetraodon

TETRAODON 8.0, Zebrafish GRCz10 and Atlantic salmon.[72] The syntenical conserved

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

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

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

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

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

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

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

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

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

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

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

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

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

FIG. 6. Kinetics of IL10 expression in resistant and susceptible carp lines during a *Trypanoplasma borreli* infection. In resistant strains, the peak of pro-inflammatory molecules expression (*iNOS, IFNγ* and *TNFα*) closely follows the increase in parasitaemia (black line). Upregulation of *IL10* (blue line) occurs in a later phase of the infection, followed by a downregulation of pro-inflammatory genes, an increase in specific antibodies, and ultimately by a reduced parasite burden. In contrast, in susceptible lines, an early expression of *IL10* is observed, prior to a very modest upregulation of pro-inflammatory genes. This leads to an uncontrolled parasite replication and increased mortalities; (Modified from Forlenza et al 2011[105] and unpublished data from our group).
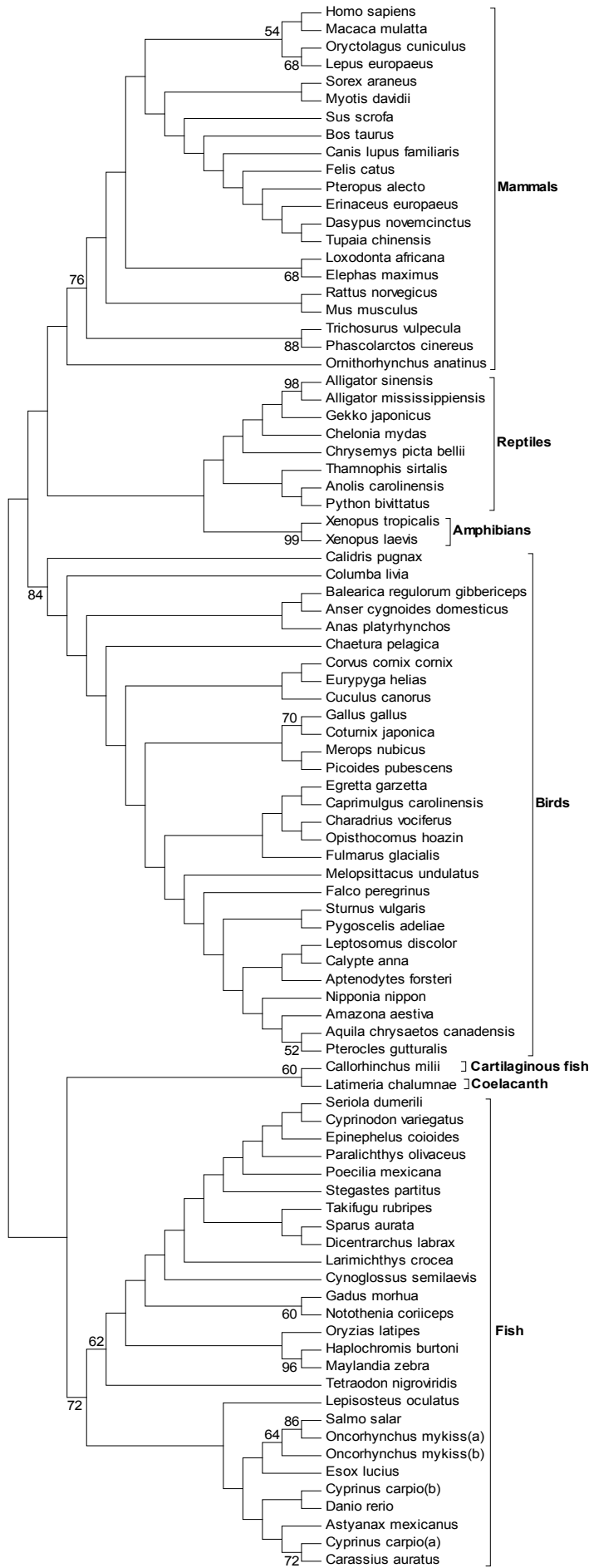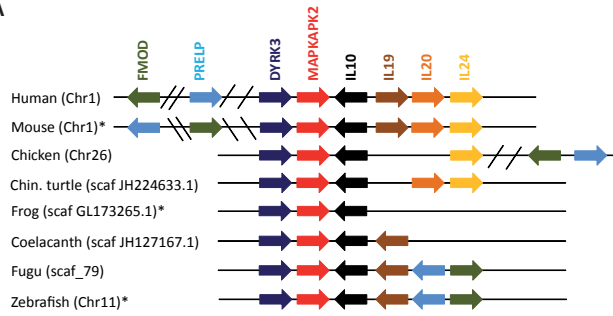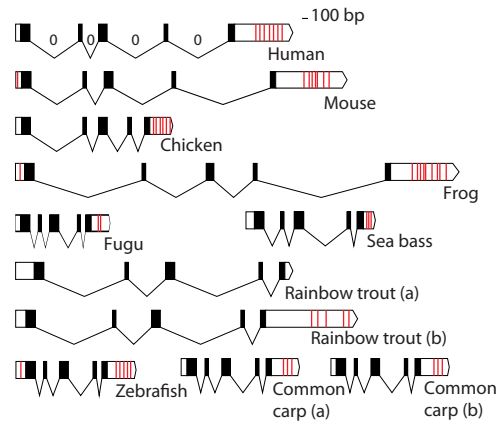
FIG 1:

Phylogenetic tree with the following taxa and groupings:

Mammals
- Homo sapiens
- Macaca mulatta — 54
- Oryctolagus cuniculus
- Lepus europaeus — 68
- Sorex araneus
- Myotis davidii
- Sus scrofa
- Bos taurus
- Canis lupus familiaris
- Felis catus
- Pteropus alecto
- Erinaceus europaeus
- Dasypus novemcinctus
- Tupaia chinensis
- Loxodonta africana
- Elephas maximus — 68
- Rattus norvegicus
- Mus musculus
- Trichosurus vulpecula
- Phascolarctos cinereus — 88
- Ornithorhynchus anatinus

(76)

Reptiles
- Alligator sinensis — 98
- Alligator mississippiensis
- Gekko japonicus
- Chelonia mydas
- Chrysemys picta bellii
- Thamnophis sirtalis
- Anolis carolinensis
- Python bivittatus

Amphibians
- Xenopus tropicalis
- Xenopus laevis — 99

(84)

Birds
- Calidris pugnax
- Columba livia
- Balearica regulorum gibbericeps
- Anser cygnoides domesticus
- Anas platyrhynchos
- Chaetura pelagica
- Corvus cornix cornix
- Eurypyga helias
- Cuculus canorus
- Gallus gallus — 70
- Coturnix japonica
- Merops nubicus
- Picoides pubescens
- Egretta garzetta
- Caprimulgus carolinensis
- Charadrius vociferus
- Opisthocomus hoazin
- Fulmarus glacialis
- Melopsittacus undulatus
- Falco peregrinus
- Sturnus vulgaris
- Pygoscelis adeliae
- Leptosomus discolor
- Calypte anna
- Aptenodytes forsteri
- Nipponia nippon
- Amazona aestiva
- Aquila chrysaetos canadensis
- Pterocles gutturalis — 52

Cartilaginous fish
- Callorhinchus milii — 60

Coelacanth
- Latimeria chalumnae

Fish
- Seriola dumerili
- Cyprinodon variegatus
- Epinephelus coioides
- Paralichthys olivaceus
- Poecilia mexicana
- Stegastes partitus
- Takifugu rubripes
- Sparus aurata
- Dicentrarchus labrax
- Larimichthys crocea
- Cynoglossus semilaevis
- Gadus morhua
- Notothenia coriiceps — 60
- Oryzias latipes
- Haplochromis burtoni
- Maylandia zebra — 96
- Tetraodon nigroviridis
- Lepisosteus oculatus
- Salmo salar — 86
- Oncorhynchus mykiss(a) — 64
- Oncorhynchus mykiss(b)
- Esox lucius
- Cyprinus carpio(b)
- Danio rerio
- Astyanax mexicanus
- Cyprinus carpio(a)
- Carassius auratus — 72

(62)
(72)

FIG 2:

FIG 3:

A Mammals, Birds and Amphibians (Reptiles?)

B Teleost fish (Coelacanth, Lungfish?)

C Duplicated genes in Teleost fish

FIG 4:

FIG 5:

FIG 6: