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The expanding repertoire of the IL-12 cytokine family in teleost fish: Identification of three paralogues each of the p35 and p40 genes in salmonids, and comparative analysis of their expression and modulation in Atlantic salmon *Salmo salar*

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ABSTRACT

Interleukin (IL)-12 family cytokines are heterodimers of an α-chain (p19, p28 and p35) and a β-chain (p40 and Ebi3), present as IL-12 (p35/p40), IL-23 (p19/p40), IL-27 (p28/Ebi3) and IL-35 (p35/Ebi3), and play key roles in immune responses in mammals. One p35 and up to three p40 genes have been cloned in some fish species. The identification of three active p35 genes, along with three p40 paralogues in salmonids in the current study further expands the repertoire of IL-12, IL-23 and IL-35 molecules in these species. The multiple p35 genes in teleost fish appear to have arisen via whole genome duplications. The different paralogues of the subunits are divergent, and differentially expressed and modulated by PAMPs and proinflammatory cytokines, hinting that distinct isoforms could be produced in response to infection. Therefore, the expanded IL-12 cytokine family may provide an unprecedented level of regulation to fine tune the immune response in fish.

1. Introduction

The mammalian interleukin (IL)-12 cytokine family consists of four heterodimeric cytokines, IL-12, IL-23, IL-27 and IL-35, that play key roles in immune responses (Watford et al., 2003; Zhang and Wang, 2008; van Wanrooij et al., 2012; Vignali and Kuchroo, 2012). Each cytokine consists of an α-chain (p19, p28 and p35) and a β-chain (p40 and Ebi3). Whilst the α subunits have a four helix bundle structure and are structurally homologous to IL-6 family cytokines (e.g. IL-6 and IL-11 etc.), the β subunits are structurally similar to the α receptors for IL-6 cytokines (e.g. IL-6Rα and CNTFR) but lack a transmembrane domain and are therefore secreted as soluble α/β heterodimers. Thus the IL-6 and IL-12 families are structurally related, forming the IL-6/12 superfamily (Jones and Vignali, 2011; Garbers et al., 2012). Chain sharing is a common feature of IL-12 family cytokines. P40 can pair with p35 and p19 to form IL-12 or IL-23, respectively, whereas Ebi3 can pair with p28 and p35 to form IL-27 and IL-35, respectively (Vignali and Kuchroo, 2012). Chain sharing extends beyond the cytokines to their receptors and signalling pathways. For example, IL-12Rβ1 can pair with IL-12Rβ2 to form the IL-12 receptor or can it pair with IL-23Rα to form the IL-23 receptor. In addition IL-12Rβ2 can pair with gp130 to form the IL-35 receptor (Vignali and Kuchroo, 2012; Collison et al., 2012).

Despite sharing many structural features and molecular partners, cytokines of the IL-12 family mediate surprisingly diverse functional effects (Vignali and Kuchroo, 2012). IL-12 and IL-23 are mainly proinflammatory and pro-stimulatory cytokines with key roles in the development of T helper (Th)1 and Th17 cells, respectively. IL-27 serves to initiate Th1 polarization but has a host of immunomodulatory activities, including limiting Th2 activity, blocking Th17 differentiation and the suppression of TGF-β-driven formation of induced regulatory T (Treg) cells and IL-10 production. IL-35 suppresses T cell proliferation and converts naive T cells into IL-35-producing induced Treg cells (iTr35 cells). Therefore a functionally balanced dichotomy in this family is established, with IL-12 and IL-23 being positive regulators and IL-27 and IL-35 being negative regulators (Vignali and Kuchroo, 2012; Collison et al., 2012).

IL-12, IL-23 and IL-27 are produced primarily by antigen presenting cells (APC), such as macrophages, dendritic cells and B cells, following detection of pathogens via pattern recognition receptors such as Toll-like receptors (TLRs). In contrast IL-35 is produced by Treg cell populations (Watford et al., 2003; Trinchieri, 2003; Zhang and Wang, 2008; Collison et al., 2012). IL-12

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production and release occurs early in the immune response following TLR recognition of pathogen associated molecular patterns (PAMPs). Production of IL-12 by dendritic cells can be initiated by TLR ligation alone, and augmented in the presence of secondary pro-inflammatory signalling. However, in macrophages, accessory signals such as IFN-γ, and IL-4 in the presence of IL-6 and TNF-α are an absolute requirement for TLR mediated induction (Kang et al., 2005; Zhang and Wang, 2008; Gee et al., 2009). To yield a bioactive heterodimer, both genes encoding the respective cytokine subunits need to be expressed co-ordinately in the same cells. Although produced by APCs, different subunits of IL-12 family members are differentially expressed and regulated by distinct signalling pathways. In response to heat-killed or viable *Salmonella enteritidis* or LPS, a characteristic mRNA expression pattern was observed in bone marrow-derived macrophages. Whereas p19 was induced early and transiently, p40 and p35 were up-regulated later and sustained continuously. p28 was also up-regulated late but declined afterwards, whereas Ebi3 remains unchanged (Schuetze et al., 2005). Signalling via TLR4 specifically promotes IL-12 production, whereas TLR2 stimulation favours the release of IL-12 and IL-23. Thus, induction of TLR-mediated signals can translate the information regarding the nature of a pathogen into differential expression of the IL-12 cytokine family and therefore contribute to the polarization of the acquired immune response (Re and Strominger, 2001).

Despite the considerable recent increase in the elucidation of the cytokine network in lower vertebrates, the fish IL-12 cytokine family remains poorly characterised (Degen et al., 2004; Secombes et al., 2011; Wang and Secombes, 2013). A single p35 gene and up to three p40 paralogues (p40a, p40b and p40c) have been reported in a few fish species (Yoshiura et al., 2003; Huising et al., 2006; Nascimento et al., 2007; Øvergård et al., 2012; Tsai et al., 2014; Wang et al., 2014). Other subunits, including Ebi3 (Secombes et al., 2011; Li et al., 2013), p19 (Holt et al., 2011) and p28 (Wang and Husain, 2013), have also been identified recently in teleost fish. Functional characterisation in rainbow trout *Oncorhynchus mykiss* showed that recombinant single chain IL-12 isoforms consisting the same p35 paralogue but different p40 paralogues can induce IFN-γ expression in head kidney (HK) cells, but possess distinct bioactivities with the respect to induction of IL-10 and p40c expression (Wang et al., 2014). Fish p40 paralogues were found to be differentially expressed and modulated *in vitro* and *in vivo* (Huising et al., 2006; Forlenza et al., 2008; Wang et al., 2014), suggesting that different IL-12 isoforms possess some conserved functions across vertebrates, such as induction of IFN-γ production (Morris et al., 1994), but have also evolved novel functions. It is unclear whether there are multiple p35 paralogues in teleost fish as due to multiple whole genome duplications (WGD) in this lineage, as seen with other fish cytokines (Husain et al., 2012; Hong et al., 2013).

Here we report the identification and characterisation of three p35 (p35a1, p35a2 and p35b) and three p40 (p40b1, p40b2 and p40c) genes in both Atlantic salmon *Salmo salar* and rainbow trout, expanding the repertoire of fish isoforms of IL-12 (i.e. p35a/p40a, p35a/p40b, p35a/p40c, p35b/p40a, p35b/p40b and p35b/p40c), IL-23 (i.e. p19/p40a, p19/p40b and p19/p40c) and IL-35 (i.e. p35a/EBI3 and p35b/EBI3). Furthermore, we produce evidence that there are two types of p35 genes present in teleosts that likely arose from the teleost-wide WGD, and that are further expanded in salmonids by their additional WGD (Macqueen and Johnston, 2014). The determination of the repertoire of the subunits of IL-12 will give a complete picture of potential isoforms of IL-12 cytokine family members, and will facilitate the functional characterisation of fish IL-12 molecules and their roles in fish immune regulation and Th cell development.

2. Materials and methods

2.1. Cloning and sequence analysis of the repertoire of salmonid IL-12 subunits

Taking advantage of the recent release of Atlantic salmon whole genome shotgun contigs (WGS), candidate WGSs for four p35 and three p40 were identified using the known trout p35 (now termed p35a1), p40b (now p40b1) and p40c (Wang et al., 2014). Homology searching was conducted using the BLAST program (Altschul et al., 1990). The exons were predicted and complete coding regions (CDS) were confirmed by PCR cloning or 3′ and 5′-RACE, as described previously (Wang and Secombes, 2003; Wang et al., 2008), using primers in Table 1. Additional trout IL-12 subunits, p35a2, p35b and p40b2 were cloned by PCR using primers (Table 1) designed against the salmon genes identified.

The nucleotide sequences generated were assembled and analysed with the AlignIR programme (Li-COR, Inc.,). Protein identification was carried out using http://www.expasy.org/tools/ (Gasteiger et al., 2005) and the presence of a signal peptide was predicted using SignalP (version 4.1) (Petersen et al., 2011). Global sequence comparison was performed using MatGAT (Campanella et al., 2003). Protein secondary structure was predicted using the Jpred3 (Cole et al., 2008) and PSIPRED programmes (Buchan et al., 2010). Multiple sequence alignments were generated using CLUSTALW (version 1.82) (Chenna et al., 2003) and shaded using BOXSHADE (version 3.21, http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed using the maximum likelihood (ML) and neighbour-joining (NJ) methods with pair-wise deletion option within the MEGA5.1 programme (Tamura et al., 2011), and were bootstrapped 10,000 times. Finally the synteny of p35 loci was analysed using Genomicus (database version 73.01) (Muffato et al., 2010).

2.2. Comparative analysis of expression of IL-12 subunits by real-time RT-PCR

The expression analysis was focused on the complete set of salmon IL-12 subunits because the expression of some subunits of trout IL-12 has been examined in a previous study (Wang et al., 2014). The primers (Table 1) for real-time-PCR were designed so that at least one primer crossed an intron, to ensure genomic DNA could not be amplified under the PCR conditions used. All primers were ordered from Eurofins MWG Operon. To directly compare the expression level of the subunits of IL-12 genes, a reference was constructed using equal mole amounts of PCR product from each gene, including the house keeping gene elongation factor-1α (EF-1α). The relative expression level of each gene was normalized against the expression level of EF-1α.

2.3. Transcript expression of the subunits of IL-12 in vivo

Atlantic salmon were maintained in freshwater or salt water fish facilities at the University of Aberdeen, UK. The water temperature was maintained at 12 °C and the fish were fed 1% body weight per day of a commercial pelleted diet (EWOS) twice daily. Six healthy salmon (average weight, 34 g) reared in fresh water aquarium were anaesthetized using phenoxyl ethanol, killed and 16 tissues (tail fin, gills, thymus, brain, scales, skin, muscle, adipose tissue, liver, spleen, gonad, head kidney, caudal kidney, intestine, heart and blood) were collected. The blood cells were washed once with PBS and dissolved in TRI reagent (Sigma). 300 mg of adipose tissue or 50 mg of other fresh tissues were directly homogenised in 1 ml of Tri reagent using a TissueLyzer II (Qiagen). The oily phase of the adipose tissue homogenate was discarded before addition of