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Differential expression of two interferon-γ genes in common carp (*Cyprinus carpio* L.)

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Summary
Two interferon gamma (IFN-γ) genes are expressed in immune cells of teleost fish and are potentially implicated in B- and T-lymphocyte responses. IFN-γ-2 shows structural and functional characteristics to other vertebrate IFN-γ genes and is associated with T-lymphocyte function. Expression profiling shows IFN-γ-2 upregulation in T-lymphocytes after phytohemagglutinin (PHA) stimulation in vitro. Unexpectedly, we found IFN-γ-1, which is structurally different from IFN-γ-2, to be expressed in lipopolysacharide (LPS)-stimulated IgM$^+$ (B- lymphocyte enriched) fractions. Expression of T-box transcription factor T-bet, but not of GATA-binding protein 3 (GATA3), correlated with expression of both IFN-γ genes. In-vivo parasite infection, but as predicted not zymosan-induced inflammation, resulted in concomitant upregulation of T-bet and IFN-γ-2. This corroborates a genuine T-lymphocyte associated role for IFN-γ-2.

Introduction

Interferon gamma (IFN-γ) is a key cytokine for innate and adaptive immunity against viral and intracellular bacterial infections and involved in tumor control. It is only active as a homodimer [1–3]. IFN-γ stimulates macrophage-mediated phagocytosis and production of pro-inflammatory cytokines and anti-microbial oxygen radicals by macrophages [4]. In mammals, IFN-γ is constitutively produced by natural killer (NK) cells of the innate arm of the immune system [5], whereas T-lymphocytes of the adaptive arm produce IFN-γ after activation or differentiation into effector T-cells in response to IL-12 and IL-18 [6]. Although different regulatory regions of the IFN-γ locus have been identified, expression is primarily regulated by two transcription factors, GATA-binding protein 3 (GATA3) and a T-cell member of the T-box family; T-bet [7,8]. T-bet is

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involved in chromatin remodeling of the IFN-γ gene to allow for IFN-γ transcription, whereas GATA3 inhibits IFN-γ expression [7,9].

The pleiotropic and redundant character of the cytokine signal family reflects its complex and subtle regulatory functions and may be at the basis of the phenomenal radiation of these signal molecules in vertebrates. Transcription factors, necessary for intracellular signaling and driving cytokine expression, execute specific functions of vital importance that elicit greater purifying selection. This is reflected by low sequence identity between cytokines of different, especially distantly related, species and high sequence conservation among transcription factors. This complicates finding orthologues of cytokine genes in representatives of evolutionary ancient species. Additionally, these orthologues might have unexpected functions.

Recently, IFN-γ was described in at least three fish species. As predicted (see above) sequence similarity is low, less than 25%, compared to mammalian IFN-γ, but the typical cytokine features, such as instability motifs, gene structure and predicted three-dimensional protein structure are comparable [10–12]. In channel catfish (*Ictalurus punctatus*), puffer fish (*Tetraodon nigroviridis*) and zebra fish (*Danio rerio*), a second IFN-γ gene was found, that may be the result from a tandem duplication [12]. In all species with duplicate IFN-γ genes, IFN-γ-2 shares more structural similarities with known vertebrate IFN-γ genes, including the human IFN-γ protein. Indeed also our common carp IFN-γ-2 shows typical features such as a comparable signal peptide, the IFN-γ signature motif ([I/V]-Q-X-[K/Q]-A-X_2-E-[L/F]-X_2-[I/V]), mRNA instability motifs and a predicted six helices secondary structure as described for human IFN-γ [13]. IFN-γ proteins have a highly and basic hydrophilic C-terminus and a nuclear localisation sequence (NLS) of four contiguous basic amino acids that are required for IFN-γ function [11]. The common carp NLS consists of four arginine residues as holds for the proteins of channel catfish, zebra fish and puffer fish [10,12]. In zebra fish and channel catfish, constitutive IFN-γ mRNA expression for both genes was demonstrated in several immune tissues and cell types and this expression is regulated by immunostimulants. In these species, IFN-γ constitutive mRNA expression of both genes was found in several immune organs and cell types, and this expression could be regulated by immune stimulants [10].

Bony fishes represent the earliest true vertebrates with a well-developed innate and adaptive immune system. The finding of two types of IFN-γ genes prompted us to search for possible ancestral functions. 'Master regulators' for IFN-γ expression, T-bet and GATA3, were recently described in T-lymphocyte-enriched lymphocyte fractions of the ginbuna crucian carp (*Carassius auratus langsdorfii*) [14,15]. We proceeded to define these genes in common carp, which gave us a unique opportunity to investigate IFN-γ function. We show differential expression of the two IFN-γ genes and the regulatory transcription factors T-bet and GATA3 by LPS- and PHA-treatments and in relation to thymocyte maturation status in vitro. Moreover, we determined expression profiles in an inflammation and an infection paradigm in vivo. Genuine IFN-γ functions were corroborated for teleost fish but interestingly, were executed by two different IFN-γ genes.

Experimental procedures

Animals

Common carp (*Cyprinus carpio* L.) were kept at 23 °C in recirculating UV-treated tap water at 'De Haar Vissen' in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7% of their estimated body weight. The cross 'R3 × R8' is offspring of Hungarian (R8) and Polish (R3) strains [16]. Experimental repeats were performed with fish from different batches of eggs. All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

Identification of IFN-γ genes

We incorporated IFN-γ-1 and IFN-γ-2 sequences described for zebra fish, channel catfish, puffer fish and rainbow trout as well as several mammalian IFN-γ sequences in separate multiple sequence alignments for IFN-γ-1 and IFN-γ-2, using CLUSTALW [17]. Primers were designed in regions of high amino acid identity. We obtained partial cDNA sequences from a λZAP cDNA library of carp brain. By rapid amplification of cDNA ends (RACE; Invitrogen, Carlsbad, CA, USA) the corresponding full-length sequences were obtained. PCR was carried out as previously described [18] and sequences were determined from both strands.

Tissue and section preparation

Nine-month-old carp (150–200 g) were anaesthetised with 0.2 g l^{-1} tricaine methane sulfonate (TMS; Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g l^{-1} $NaHCO_3$ (Merck, Darmstadt, Germany). Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe fitted with a 21-Gauge needle. Next, fish were killed by spinal transection and organs and tissues for RNA extraction were carefully removed, snap frozen in solid CO_2 or liquid N_2 and stored at −80 °C.

Cell collection

Gill and gut lymphocytes: Intestinal and branchial epithelia were collected by scraping the epithelia off the underlying connective tissue and branchial arches, respectively, on an ice-cooled glass plate using a microscope slide. The tissues thus obtained were passed through a 100 μm nylon mesh (BD Bioscience, Breda, The Netherlands) with carp RPMI (cRPMI, 280 mOsm) and washed twice. The cell suspension was layered on 3 ml Ficoll (density 1.077 g ml^{-1}; Amersham Biosciences, Uppsala, Sweden). Following subsequent centrifugation at 800*g* at 4 °C for 25 min with the brake disengaged, leukocytes at the interface were collected and washed twice with cRPMI and once with $cRPMI^{++}$ (cRPMI suplemented with 0.5% pooled carp serum, 1% glutamine (Cambrex, Verviers, Belgium), 1% penicillin G (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1% streptomycin sulphate (Sigma-Aldrich).