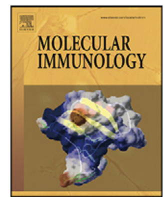
Molecular Immunology 48 (2011) 1073–1083

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Evolutionary conserved pro-inflammatory and antigen presentation functions of zebrafish IFNγ revealed by transcriptomic and functional analysis

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ARTICLE INFO

Article history:
Received 2 December 2010
Received in revised form 26 January 2011
Accepted 31 January 2011
Available online 26 February 2011

Keywords:
Neutrophils
Macrophages
Immunoproteasome
Myelopoiesis
Comparative immunology/evolution

ABSTRACT

In mammals, IFNγ is the only type II IFN member, whereas most bony fish species have two IFNγ genes, namely IFNγ1 and IFNγ2. We report that both zebrafish IFNγ genes were unable to protect zebrafish larvae against viral infection, despite the fact that they moderately induced the expression of antiviral genes, strongly induced pro-inflammatory and antigen processing and presentation genes, and increased neutrophil numbers. Although both zebrafish IFNγs induced a similar set of immune genes, IFNγ1 was more powerful at inducing pro-inflammatory genes than IFNγ2, which correlated with its ability to promote larval death. Strikingly, IFNγ1-induced larval death was prevented by genetic ablation of the myeloid transcription factor SPI1 but not IL-1β or TNFα, suggesting that professional phagocytes are also one of the main targets of IFNγ in fish. In addition, the usefulness of the zebrafish for the identification of IFNγ-target genes is illustrated by the identification of several genes whose expression is also regulated in murine macrophages by IFNγ, namely two membrane-spanning 4-domain family members and the opioid growth factor receptor. Finally, we found for the first time that the thymic specific proteasome subunit PSMB11/β5t is regulated by IFNγ. Collectively, our data throw light on partially redundant functions of fish IFNγ genes, demonstrate that the pro-inflammatory and antigen presentation functions of IFNγ have been conserved during vertebrate evolution, and highlight the fact that zebrafish is an excellent model for studying IFNγ biology.

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1. Introduction

IFNγ is a cytokine implicated in both innate and adaptive immune responses. During innate responses it is produced constitutively by NK cells (Stetson et al., 2003) and in adaptive responses by Th1 lymphocytes and CTLs in response to MHC-presented antigens (Robertsen, 2006). IFNγ upregulates the expression of genes involved in antigen processing and presentation, and stimulates phagocytosis and the production of pro-inflammatory cytokines and reactive oxygen species (ROS) by macrophages (Schoenborn and Wilson, 2007). However, IFNγ is also able to limit tissue damage due to inflammation and to modulate T cell proliferation and differentiation (Hu and Ivashkiv, 2009).

In mammals, IFNγ is the only type II IFN member, whereas most bony fish species, such as channel catfish (*Ictalurus punctatus*, Siluriformes) (Milev-Milovanovic et al., 2006), pufferfish (*Tetraodon nigroviridis* and *Takifugu rubripes*, Tetraodontiformes) (Igawa et al., 2006), common carp (*Cyprinus carpio*, Cypriniformes) (Stolte et al., 2008), goldfish (*Carassius auratus*, Cypriniformes) (Grayfer and Belosevic, 2009a,b; Grayfer et al., 2010) and zebrafish (*Danio rerio*, Cypriniformes) (Igawa et al., 2006), present two IFNγ genes, namely IFNγ1 (also known as IFNγ1-1 and IFNγrel) and IFNγ2 (also known as IFNγ1-2). As in mammals, fish IFN-γ genes present 4 exons and three introns placed at similar sites to those in its human homolog (Igawa et al., 2006). Notably, fish IFNγ genes share a low amino acid identity (17%) and the amino acid similarity/identity with mammalian IFNγ is low (less than 25%), although in the case of IFNγ2 it is more similar. However, both IFNγ genes conserve typical features of mammalian IFNγ, including the signal peptide, signature motif, mRNA instability motifs and a predicted

Abbreviations: B2M, β-2-microglobulin; COX-2, cyclooxygenase-2; dpf, days post-fertilization; dpi, days post-injection; EPC, epithelioma papulosum cyprinid; hpi, hours post-injection; HSCs, hematopoietic stem cells; ISGs, IFN-stimulated genes; IRF, IFN regulatory factor; MMP, metalloproteinase; Mo, morpholino; Mx, myxovirus (influenza virus) resistance; MS4, membrane-spanning 4-domain; OGFR, opioid growth factor receptor; PSMB, proteasome (prosome macropain) subunit β; ROS, reactive oxygen species; SOCS, suppressor of cytokine signaling; SPI1, spleen focus forming virus proviral integration oncogene; SVCV, spring viremia of carp virus; TCID, tissue culture infection dose; zfIFN, zebrafish IFN.

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doi:10.1016/j.molimm.2011.01.015

secondary structure of six helices (Igawa et al., 2006; Stolte et al., 2008). Strikingly, however, IFNγ1 lacks a nuclear localization signal, which is needed for the biological activity of mammalian IFNγ. Finally, their expression pattern is also different: both are expressed in typical immune organs, such as head kidney, spleen or intestine, and immune cells but IFNγ2 expression is usually higher than that of IFNγ1 (Milev-Milovanovic et al., 2006; Grayfer et al., 2010). More interestingly, in common carp IFNγ2 gene expression is induced in T lymphocytes after PHA stimulation, while IFNγ1 is induced in B lymphocytes upon LPS stimulation (Stolte et al., 2008), suggesting a genuine T-lymphocyte associated role only for IFNγ2.

The biological activity of fish IFNγ has began to be elucidated. While IFNγ2 shows conserved macrophage antimicrobial activity in phylogenetically distant species (Zou et al., 2005; Grayfer and Belosevic, 2009a,b; Arts et al., 2010; Grayfer et al., 2010), it fails to induce the expression of antiviral and pro-inflammatory genes in zebrafish when administered *in vivo*, which correlates with its inability to protect the fish against bacterial and viral infections (López-Muñoz et al., 2009). As regards IFNγ1, its biological function is controversial, since it has been shown that recombinant carp IFNγ1 is unable to induce iNOS in macrophages (Arts et al., 2010), while goldfish IFNγ1 displays a stronger macrophage antimicrobial activity than IFNγ2 (Grayfer et al., 2010). Complementary rather than overlapping functions are expected, taking into account that they signal through different receptors, show very low homology and only IFNγ2 has a canonical nuclear localization signal (Grayfer and Belosevic, 2009b). Therefore, a great deal remains to be known about the biological activity of fish IFNγ genes and whether they play complementary or redundant functions *in vivo*. Using the genetically tractable zebrafish model, we report in this study that both zebrafish IFNγs were unable to protect zebrafish larvae from viral infection, although they strongly induced pro-inflammatory and antigen processing and presentation genes. While both IFNγ genes induced a similar set of immune genes, IFNγ1 was more powerful at inducing pro-inflammatory genes than IFNγ2, which correlated with its ability to promote the death of young larvae. Strikingly, IFNγ1-induced larval death was prevented by genetic ablation of the myeloid transcription factor SPI1 (PU1) but not by knockdown of the pro-inflammatory cytokines IL-1β and TNFα. Collectively, our data point to the partially redundant functions of fish IFNγ genes and, more importantly, demonstrate that the pro-inflammatory and antigen presentation functions of IFNγ are conserved from fish to mammals. The usefulness of the zebrafish model for identifying genes whose expression is regulated in mammalian macrophages by IFNγ is also illustrated.

2. Materials and methods

2.1. Animals

Wild-type zebrafish (*D. rerio* H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC) and maintained as described in the zebrafish handbook (Westerfield, 2000). The transgenic zebrafish line that expresses enhanced GFP driven by the neutrophil-specific promoter for the myeloid-specific peroxidase (*mpx*) gene (Tg(*mpx*::gfp)^{i114}) has been described previously (Renshaw et al., 2006).

Eight-week old mice (*Mus musculus* L.) of the Balb/c strain were maintained in the Animal Facility of the University of Murcia with a commercial diet and water *ad libitum*. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the Bioethical Committee of the University of Murcia (approval number #333/2008) for the use of laboratory animals.

2.2. Expression constructs

zfIFNγ1 (NM_001020793) were obtained by PCR amplification using a proof-reading DNA polymerase (*Pfu*, Fermentas), cDNA from spring viremia of carp virus (SVCV)-infected zebrafish as a template and the PCR primers indicated in Supplementary Table 1. The PCR-amplified fragments were incubated at 72 °C for 10 min with 1 unit of Taq DNA polymerase (Invitrogen) for the addition of 3′ A-overhangs and cloned into a pcDNA3.1/V5-His-TOPO vector (Invitrogen) for the expression of V5/His6-tagged proteins. The construct was sequenced using an ABI PRISM 377 (Applied Biosystems). zfIFNγ2 (AB158361) construct was also in pcDNA3.1/V5-His backbone and has previously been described (López-Muñoz et al., 2009).

2.3. Morpholinos and microinjection

Splice- or translation-blocking MOs were designed by and purchased from Gene Tools (Supplementary Table 2) and solubilized in water (1–3 mM). Plasmid DNA (100 pg/egg) and MOs (1–8 ng/egg) were mixed in microinjection buffer (0.5× Tango buffer and 0.05% phenol red solution) and microinjected (0.5–1 nl) into the yolk sac of one-cell-stage embryos using a Narishige IM300 microinjector.

2.4. Infection assays

The SVCV isolate 56/70 was kindly provided by Dr. P. Fernández-Somalo (Laboratorio Central de Veterinaria, MARM). The virus stock was propagated in EPC cells and titrated to 96-well plates according to Reed and Muench (Reed and Muench, 1938). Groups of 25–30 zebrafish larvae were challenged 3 days post-fertilization (dpf) in 5 ml egg water (60 μg/ml sea salts in distilled water) containing from 2.5×10^7 to 10^8 50% tissue culture infectious dose ($TCID_{50}$)/ml SVCV at 26 °C. Twenty four hours later, the virus was diluted by adding 35 ml of egg water and the larvae were monitored every 24 h over a 10-day period for clinical signs of disease and mortality (López-Muñoz et al., 2010). In addition, 24 and 48 h post-infection (hpi), 10 larvae were collected per experimental group, pooled and processed for analysis of gene expression (see below).

2.5. Microarray analysis

Total RNA was extracted from about 100 pooled larvae at 4 dpf with TRIzol® Plus RNA Purification System (Invitrogen) following the manufacturer's instructions. RNA amplification and labeling and statistical data analysis were performed basically as previously described (Adie et al., 2007). The slides used were Agilent Zebrafish Oligo Microarrays (reference #019161), which allows the analysis of four biological replicates. For each hybridization, the protocol used was that published by Agilent Technologies, in particular "The manual two color microarray based gene expression Analysis" (version 5.7). Images from Cy3 and Hyper5 channels were equilibrated and captured with a GenePix 4000B (Axon) and spots were quantified using GenPix software (Axon).

Background correction and normalization of the expression data were performed using LIMMA (Smyth and Speed, 2003; Smyth, 2004) which is part of Bioconductor, a R language project (Ihaka and Gentleman, 1996). First, the data set was filtered according to spot quality. A strategy of adaptive background correction was used to avoid excessive variability of log-ratios for low-intensity spots. For local background correction, the "normexp" method in LIMMA was used to adjust the local median background. The resulting log-ratios were print-tip lowess normalized for each array (Smyth and Speed, 2003). To achieve a similar distribution across arrays and consistency among arrays, log-ratio values were scaled using the median-absolute-value as scale estimator (Smyth and

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