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New Insights into the Evolution of IFNs: Zebrafish Group II IFNs Induce a Rapid and Transient Expression of IFN-Dependent Genes and Display Powerful Antiviral Activities

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The IFNs and their receptors have existed in early chordates for ~500 million years and represent the early elements in innate and adaptive immunity. Both types I and II IFNs have been discovered in fish, and type I has recently been classified into two groups based on their primary protein sequences. However, the biological activities of fish IFNs and their roles in infection are largely unknown. Using the zebrafish and manageable bacterial (Streptococcus iniae) and viral (spring viremia of carp virus) infection models, we are reporting in this study that zebrafish IFN (zfIFN) γ failed to induce antiviral and proinflammatory genes when administered in vivo, which correlates with its inability to protect the fish against bacterial and viral infections. We also found that, although both group I (i.e., zfIFN1) and group II zfIFNs (i.e., zfIFN2 and zfIFN3) displayed strong in vivo antiviral activities, only group I zfIFN was able to protect the fish against bacterial infection, which may reflect the different patterns and kinetics of immune-related genes elicited by these two groups of IFNs. Thus, group II zfIFNs induced a rapid and transient expression of antiviral genes, whereas group I zfIFN exerted a slow but more powerful induction of several antiviral and proinflammatory genes. Collectively, our results suggest nonredundant, complementary roles of type I zfIFNs in viral infections and provide evidence for a pivotal role of the recently identified group II IFN of fish in the early stages of viral infections. The Journal of Immunology, 2009, 182: 3440–3449.

The IFNs and their receptors represent a subset of class 2 α-helical cytokines that have existed in early chordates for ~500 million years and represent early elements in innate and adaptive immunity (1). Approximately 10 mammalian IFN species have been discovered, and they are classified into type I (IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNτ, IFNς, and IFNζ), type II (IFN-γ), and type III (IFNλ) (1, 2). As a major component of the innate immune system protecting against viral infection, the expression of type I and type III IFNs is induced by viral challenges, and the TLRs play an important role in their expression (3). In contrast, type II IFN-γ is secreted by T lymphocytes under certain conditions of activation and by NK cells (4). Although originally defined as an agent with direct antiviral activity, the properties of IFN-γ include the regulation of several aspects of the immune response, such as the stimulation of bactericidal activity of phagocytes, the stimulation of Ag presentation through class I and class II MHC molecules, the orchestration of leukocyte-endothelium interactions, effects on cell proliferation and apoptosis, as well as the stimulation and repression of a variety of genes (4, 5).

IFNs are restricted to vertebrates and have been studied mainly in birds and mammals (2). More recently, type II IFN-γ has been sequenced in several teleost fish, found to be relatively well-conserved, and, interestingly, some teleosts have more than one IFN-γ gene (6). Functional studies in the rainbow trout (Oncorhynchus mykiss, order Salmoniformes) have shown that IFN-γ expression is induced in the head kidney (bone marrow equivalent of fish) and the spleen of fish injected with polyinosinic-polycytidylic acid and that the recombinant protein stimulates gene expression IFN-γ-inducible protein γIP, MHC class II β-chain, and STAT1 and enhanced respiratory burst activity in macrophages (7). Similarly, goldfish (Carassius auratus, order Cypriniformes) IFN-γ primes macrophages and neutrophils for enhanced respiratory burst responses and increases the phagocytic and NO responses of macrophages (8). In addition, virus-induced IFNs have also been identified in teleost fish (9), but their designation has been controversial. Although some authors have designated these virus-induced IFNs as type III IFNA based on the genomic organization of the zebrafish IFN gene (10) and on the protein structure of its receptor components (11), others have designated them as type I IFN on the basis of the primary nucleotide and protein homology, their cysteine pattern, and the presence of a CAWE motif, typical of mammalian IFNαs (12). In addition, fish virus-induced IFNs have recently been classified into two groups based on their primary protein sequences: group I containing two cysteine residues and universally present in teleosts, and group II containing four cysteine residues and found only in more primitive species such as the rainbow trout and zebrafish (Danio rerio, order Cypriniformes) (12). These two groups differ in their expression pattern and biological activities. Notably, group I IFNs from the zebrafish (13), the rainbow trout (12), the Atlantic salmon (Salmo salar, order Salmoniformes) (14), and the channel catfish (Ictalurus punctatus,
order Siluriformes) (15) were potent at inducing myxovirus (influenza virus) resistance (Mx)3 gene expression and eliciting antiviral responses in cell lines, whereas the group II IFN from the rainbow trout was poor in these activities (12). In addition, two recent studies have also shown that zebrafish group I IFN increases the survival of embryos infected with spring viremia of carp virus (SVCV) (11) and the survival of rainbow trout fingerlings infected with infectious hematopoietic necrosis virus (16). To date, however, the in vivo relevance of virus-induced fish IFNs in bacterial and viral infections is poorly understood, whereas that of group II IFNs and IFN-γ is completely unknown. To get insights into the fish IFN functions, we set up both bacterial and viral infection models in the zebrafish and tested the bioactivities of recombinant zebrafish IFNs (zfIFNs). Our results show that although all virus-induced zfIFNs displayed powerful in vivo antiviral activities, only group I zfIFN was able to protect the fish against bacterial infection. However, IFN-γ had negligible effects on the resistance of zebrafish to infection. Finally, the immune gene expression profiles induced by zfIFNs revealed that group II zfIFNs induced a rapid and transient expression of antiviral genes, whereas group I zfIFN exerted a slow but more powerful induction of several antiviral and proinflammatory genes. Collectively, our results suggest nonredundant, complementary roles for virus-induced zfIFNs in viral infections and provide evidence of a pivotal role for the recently identified group II IFNs of fish in the early stages of viral infections.

Materials and Methods

Animals

Wild-type zebrafish (Danio rerio, order Cypriniformes, family Cyprinidae) were obtained from the Zebrafish International Resource Center and maintained as described by M. Westerfield (17). All animal studies were conducted in accordance with the European Union regulations for animal experimentation.

Expression constructs

zfIFN1 (GenBank accession no. NM_207640), zfIFN2 (GenBank accession no. NC_007114), zfIFN3 (GenBank accession no. NC_007114) and zfIFNγ-1–2 (GenBank accession no. AB158361) were obtained by PCR amplification with a proof-reading DNA polymerase (Pfu; Fermentas) using cDNA from SVCV-infected zebrafish as a template and the PCR primers indicated in Table I. The PCR-amplified fragments were incubated at 72°C for 10 min with 1 U 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. All constructs were sequenced by using an ABI PRISM 377 (Applied Biosystems).

Production of zebrafish recombinant IFNs

Plasmid DNA was prepared using the Midi-Prep procedure (Qiagen). DNA pellets were resuspended in water and further diluted when required. In PBS, Transfections were performed with a cationic lipid-based transfection reagent (Fermentas) according to the manufacturer’s instructions. Briefly, HEK293 cells were plated in 6-well plates (400,000 cells per well) together with 100 μl of transfection reagent containing 1 μg of plasmid DNA. Forty-eight hours after the transfection, supernatants were collected, clarified with a 0.45-μm filter, concentrated with a Microcon 10-kDa molecular mass cutoff filter unit (Millipore) and stored in small aliquots at −80°C. For the deglycosylation experiments, supernatants were incubated with 0.45-μm filter, concentrated with a Microcon 10-kDa molecular mass cutoff filter unit (Millipore) and stored in small aliquots at −80°C. For the deglycosylation experiments, supernatants were collected and processed for analysis of gene expression (see below).

Infection assays

The SVCV isolate 56/70 was provided by Dr. P. Fernández-Somalo (Laboratorio Central de Veterinaria, Ministerio de Medio Ambiente y Medio Rural y Marino, Algecete, Spain). The virus stock was propagated in EPC cells and titered in 96-well plates according to Reed and Muench (18). The strain REFP 404 (serotype II) of Streptococcus iniae was provided by Dr. A. Eldar (The Hebrew University-Hadassah Medical School, Jerusalem, Israel) and cultured in brain/heart infusion broth as described previously (19). Ten adult zebrafish were challenged at 26°C in 10-liter tanks i.p. with 104 50% tissue culture infection dose (TCID50)/ml SVCV or 104 CFU/ml S. iniae (20) alone or combined with 5 μl of supernatants from HEK293 cells containing recombinant zfIFNs. Supernatants from HEK293 cells transfected with the empty plasmid were used as control. Four, 20, 30, and 72 h postinjection, the heads from four fish were collected and processed for the analysis of gene expression (see below).

Analysis of gene expression

Total RNA was extracted from tissues with TRIzol Reagent (Invitrogen) following the manufacturer’s instructions and treated with DNase I, amplification grade (1 μg/ml RNA; Invitrogen). The SuperScript III RNase H reverse transcriptase (Invitrogen) was used to synthesize the first strand of cDNA with an oligo(dT)18 primer from 1 μg of total RNA at 50°C for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C. For each mRNA, the gene expression was calculated relative to the ribosomal protein S11 content in each sample using the comparative Ct method (2−ΔΔCt) (where Ct is cycle threshold). The primers used are shown in Table I. In each case, PCR was performed with triplicate samples and repeated with at least two independent samples.

Statistical analysis

Data were analyzed by ANOVA and a Tukey multiple range test to determine the differences between groups. A log rank test was used to calculate the statistical differences in the survival of the different experimental groups.

Results

Production of recombinant zfIFNs in HEK293 cells

HEK293 cells were transiently transfected with the different expression plasmids of zfIFNs and the production of the recombinant

3 Abbreviations used in this paper: Mx, myxovirus (influenza virus) resistance; dpi, days postinfection; EPC, epitheliolema papulosum cyprinid; hpi, hours postinfection; NOS, NO synthase; PKZ, protein kinase containing Z-DNA binding domain; SVCV, spring viremia of carp virus; TCID50, 50% tissue culture infection dose; zfIFN, zebrafish IFN.

a specific mAb to the V5 epitope (Invitrogen) and developed with ECL reagents (GE Healthcare) according to the manufacturer’s protocol.

Cell culture and treatments

The zebrafish embryonic cell line ZF4 was purchased from the American Type Culture Collection, whereas the epitheliolema papulosum cyprinid (EPC) cell line was provided by Dr. A. Estepa (Miguel Hernández University, Elche, Spain). ZF4 cells were maintained at 28°C in DMEM/F12 culture medium (Invitrogen) supplemented with 10% FCS, 15 mM HEPES, 0.5 mM sodium pyruvate (Sigma-Aldrich), and 100 IU/ml penicillin plus 100 μg/ml streptomycin (Biochrom), whereas the EPC cells were grown at 25°C in DMEM (Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 μg/ml amphotericin B (Biochrom), and 50 μg/ml gentamicin (Biochrom). ZF4 cells were pretreated for 2 h with different dilutions of recombinant zfIFNs before being infected with 4×104 or 4×105% tissue culture infection dose (TCID50/ml SVCV, incubated for 48 h at 25°C in the presence of zfIFNs, and then fixed and stained for 2 h in a solution containing 4% paraformaldehyde, 1% crystal violet, and 0.9% NaCl. The number and area of the plaques were visualized using a Nikon inverted microscope (Eclipse TE2000) and analyzed with the MIP-4.5 image analysis software (Digital Image System).

Injection of fish with recombinant zfIFNs

Adult zebrafish were anesthetized by immersion in benzocaine (100 μg/ml) (Sigma-Aldrich) before injection. Each fish was injected i.p. with 5 μl of supernatants from HEK293 cells containing recombinant zfIFNs. Supernatants from HEK293 cells transfected with the empty plasmid were used as control. Four, 20, 30, and 72 h postinjection, the heads from four fish were collected and processed for the analysis of gene expression (see below).

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Statistical analysis

Data were analyzed by ANOVA and a Tukey multiple range test to determine the differences between groups. A log rank test was used to calculate the statistical differences in the survival of the different experimental groups.
Table I. Primers used in this study

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a The gene symbols followed the Zebrafish Nomenclature Guidelines (zfin.org/zf_info/nomen.html).
b F, Forward; R, reverse.
c ORF, Open reading frame.
proteins were analyzed in cell extracts (data not shown) and super- 

ternants (Fig. 1) by means of Western blotting using the V5 m Ab. All zfIFNs were satisfactorily expressed and efficiently secre- 
ted. The supernatants were concentrated through 10-kDa mole- 

cular mass cutoff centrifugal filter units and the recombinant zfIFNs were adjusted to similar concentrations (Fig. 1). zfIFN2, 

zfIFN3, and zfIFNγ showed higher molecular mass species that 

may be the result of an extensive glycosylation during the secre- 
tory pathway. Therefore, we treated recombinant zfIFNs with en- 
doglycosidase F2 and found that zfIFN1, zfIFN2, and zfIFNγ are 
sensitive to this treatment whereas zfIFN3 was largely resistant 
(Fig. 1B). This might suggest that zfIFN3 underwent other post- 
translational modifications or that it was resistant to deglycosyla- 
tion by endoglycosidase F2. Importantly, all recombinant zfIFNs 
were biologically active because they were able to fully protect 
ZF4 cells against SVCV infection (Fig. 2A). In addition, zfIFN1 
and zfIFNγ were able to induce the expression of MxB and MxC 
in ZF4 cells (Fig. 2B), which might explain their ability to protect 
these cells against SVCV infection.

zfIFN1 is able to protect zebrafish from S. iniae infection

We first tested the role played by zfIFNs in bacterial infections by 

using a S. iniae infection model (20) and found that only group I 

IFNs, i.e., zfIFN1, was able to protect the fish against this pathogen 

(Fig. 3). No mortality was observed in fish injected with the zfIFNs 
alone for up to 7 days (data not shown). To understand the innate 
immune response of the zebrafish to S. iniae infection, we analyzed 

FIGURE 1. Production of recombinant zfIFNs in HEK293 cells. A. 

HEK293 cells were transfected with the different V5/His-tagged zfIFN 

expression constructs. Forty-eight hours after transfection, the supernatants were collected, concentrated through 10 molecular mass cutoff filter units, and probed with the V5 m Ab. B. The concentrated supernatants containing 

zfIFNs were incubated for 2 h at 37°C without (−) or with (+) 2 μl of 

endoglycosidase F2 (EndoF2) and then probed with the V5 m Ab. The deglycosylated zfIFNs were indicated with arrowheads.

FIGURE 2. zfIFNs protect ZF4 cells against SVCV infection. A. Representative phase-contrast micrographs of ZF4 cell monolayers 48 h after being infected with 4 × 10⁴ TCID₅₀/ml SVCV. Cells were pretreated for 2 h with a 1:10 dilution of supernatants from HEK293 cells containing the indicated 

zfIFNs. Noninfected cells (−SVCV) and cells pretreated with supernatants from HEK293 transfected with the empty plasmid (+SVCV) are also shown. The number of plaques/well (mean ± SE) is indicated in each treatment. B. The mRNA levels of mxb and mxc were determined by real-time RT-PCR in ZF4 cells incubated for 4 and 16 h with a 1:10 dilution of supernatants from HEK293 cells containing recombinant zfIFN1 (left panel) and zfIFNγ (right panel). Gene expression is normalized against rps11 and is shown as relative to the mean of control cells. Each bar represents the mean ± SE of triplicate 

samples. Different letters (a and b) denote statistically significant differences between the groups according to Tukey’s test.
**FIGURE 3.** zfIFN1 protects zebrafish from *S. iniae* infection. A, Survival of zebrafish challenged i.p. with 10³ CFU/fish *S. iniae* alone or in combination with 5 μL of concentrated supernatants from HEK293 cells containing recombinant zfIFNs. The supernatants from HEK293 cells transfected with the empty expression plasmid were used as control. Each infection was performed with 10 fish and the data represent the mean of two independent experiments (n = 20 fish/treatment). The number of survivors relative to the number of animals in each group is indicated between brackets following each survival curve. B and C, The mRNA levels of the genes coding for the indicated antiviral, proinflammatory, anti-inflammatory, and apoptotic molecules were determined by real-time RT-PCR in the heads of zebrafish challenged with 10⁶ CFU/fish live (B) or heat-killed (C) *S. iniae* at the indicated times p.i. The gene expression is normalized against *rps11* and is shown as relative to the mean of nonchallenged fish. Each bar represents the mean ± SE of four fish. Different letters denote statistically significant differences between the groups according to a Tukey’s test. The groups marked with “a” did not show statistically significant differences from nonchallenged fish.
**FIGURE 4.** zfIFN1, zfIFN2 and zfIFN3 protect zebrafish from SVCV infection. A. Survival of zebrafish challenged i.p. with $10^5$ TCID$_{50}$/fish SVCV alone or in combination with 5 µl of supernatants from HEK293 cells containing recombinant zfIFNs. The supernatants from HEK293 cells transfected with the empty expression plasmid were used as control. Each infection was performed with 10–15 fish and the data represent the mean of two independent experiments ($n = 20–30$ fish/treatment). The number of survivors relative to the number of animals in each group is indicated between brackets following each survival curve. B and C. The mRNA levels of the genes coding for the indicated antiviral, proinflammatory, anti-inflammatory, and apoptotic molecules were determined by real-time RT-PCR in the heads of control and SVCV-challenged zebrafish at the indicated postinjection times (B) or in symptomatic and asymptomatic fish 4 dpi (C). The mRNA levels of the gene coding for the SVCV N protein was also determined as an estimation of the viral load in the infected tissues. The gene expression is normalized against *rps11* and is shown as relative to the mean of nonchallenged fish (A) or as the ratio of symptomatic vs asymptomatic fish (C). Each bar represents the mean ± SE of four fish. B, Different letters denote the statistically significant differences between the groups according to a Tukey’s test. The groups marked with “a” did not show statistically significant differences from nonchallenged fish. C, The asterisks (*) denote the statistically significant differences between symptomatic and asymptomatic fish.
the expression levels of zfIFNs and other immune- and apoptosis-related genes upon infection. Surprisingly, injection of zebrafish with $10^3$ CFU of live or heat-killed *S. iniae* per fish did not affect the mRNA levels of the genes analyzed (data not shown), whereas a higher dose ($10^6$ CFU/fish) of live or heat-killed bacteria exerted a weak effect on the expression levels of most genes with the exception of IL-1β, which considerably increased 20 hpi of live bacteria (Fig. 3, B and C). Notably, the mRNA levels of caspase 9

**FIGURE 5.** IFN1 drastically induces the expression of antiviral and proinflammatory genes. The mRNA levels of the indicated genes were determined by real-time RT-PCR in the heads of zebrafish injected with 5 μl of control supernatants from HEK293 cells or containing recombinant zfIFN1 (A), zfIFNγ (B), and zfIFN1 plus zfIFNγ (C). The gene expression is normalized against *rps11* and presented as mean ± SE of four fish. Different letters denote the statistically significant differences between the groups according to a Tukey’s test. The groups marked with “a” did not show statistically significant differences from fish injected with control supernatants.
Increased at similar rates in zebrafish injected with live and heat-killed bacteria, whereas those of caspase 3 did not significantly change (Fig. 3, B vs C). These results seem to suggest that the zebrafish is unable to mount an immune response to S. iniae, which might facilitate its rapid dissemination and high virulence. However, the administration of zfIFN1 is sufficient to facilitate the clearance of this bacterium.

**zfIFN1, zfIFN2, and zfIFN3 are able to protect zebrafish from SVCV infection**

We next evaluated the in vivo antiviral activities of zfIFNs by using an SVCV infection model (20). Interestingly, both group I and II virus-induced zfIFNs were able to increase the resistance of zebrafish to SVCV, whereas zfIFNγ showed a no statistically significant effect on the susceptibility of zebrafish to this virus when injected alone or combined with zfIFN1 (Fig. 4A). Higher concentrations of recombinant zfIFNs showed similar effects (data not shown).

To understand the response of zebrafish to SVCV, we analyzed the expression profile of several antiviral and immune-related genes in SVCV-infected zebrafish. The results showed that the infection drastically induced the mRNA levels of most antiviral and immune-related genes 24 and 48 hpi, including all zfIFNs, MxC, RSAD2 (Vig1/Viperin), protein kinase containing Z-DNA binding domains (PKZ), STAT1, TNF-α, lymphotoxin α, IL-1β, and IL-12 (Fig. 4B). However, the mRNA levels of the anti-inflammatory genes IL-10 and TGFβ1 were unaffected by the infection (Fig. 4B). As 30–40% of control fish were always able to survive the infection, we analyzed the cytokine expression and viral load in symptomatic vs asymptomatic fish at a critical time after infection (4 days postinjection (dpi)) to clarify whether zfIFNs were responsible for this resistance. The results showed unexpected higher mRNA levels of most antiviral genes (zfIFN1, zfIFN2, zfIFN3, zfIFNγ, MxB, MxC, and RSAD2) as well as of those coding for proinflammatory (IL-1β and IL-12) and anti-inflammatory (IL-10) cytokines in symptomatic fish (Fig. 4C). Moreover, the viral load, assayed as the mRNA levels of the N protein of the virus, was also significantly higher in symptomatic fish compared with their asymptomatic littermates (Fig. 4C). These results might suggest that the SVCV is able to interfere in the
translation of zfIFN mRNAs and/or that a high production of zfIFNγ is detrimental for the resolution of SVCV infection.

**Group II virus-induced IFNs induce a rapid expression of antiviral genes**

As the potent in vivo antiviral activity of group II zfIFNs sharply contrasts with the poor antiviral activity of this group of IFNs from the rainbow trout when assayed in the RTG-2 cell line (12), we evaluated the immune gene expression profiles of zebrafish infected with the different recombinant zfIFNs. zfIFN1 induced a strong but slow (48 and 72 hpi) increased expression of all antiviral genes analyzed such as zfIFNs, MxB, MxC, RSAD2, PKZ, and STAT1, as well as those coding for proinflammatory IL-1β and IL-12 (Fig. 5A). In contrast, zfIFNγ had a weak effect on the expression of the gene analyzed (Fig. 5B) and ameliorated the effects of zfIFN1 when they were administered together (Fig. 5C). However, the combination of zfIFN1 and zfIFNγ resulted in a quicker induction of antiviral and proinflammatory genes when compared with zfIFN1 alone (Fig. 5, A vs C). These unexpected negligible effects of zfIFNγ on immune gene expression, together with the strong inducibility of murine inducible NO synthase (NOS) 2 by IFN-γ (21), led us to analyze the expression of the two genes coding for the zebrafish NOS2 and found that zfIFN1, rather than IFN-γ, increased 30 hpi the mRNA levels of NOS2b (Fig. 5, A vs B).

Finally, we studied the effects of the administration of group II virus-induced zfIFNs in the immune gene expression profile of zebrafish. Strikingly, both zfIFN2 (Fig. 6A) and zfIFN3 (Fig. 6B) were capable of increasing the mRNA levels of MxB but not of MxC, although zfIFN3 was considerably more potent than zfIFN2. In addition, zfIFN2 also increased the mRNA levels of IL-1β, whereas zfIFN3 strongly increased those of the genes coding for the antiviral proteins Vig1/Viperin (RSAD2 gene) and PKZ. More importantly, all of these effects were rapidly and transiently observed (i.e., 4 and 20 hpi), suggesting complementary roles to group I zfIFN.

**Discussion**

Although IFN genes have been cloned in some teleost fish, the functions displayed by these cytokines in lower vertebrates remain poorly understood. In addition, the fish IFN system seems to be as complicated as that of mammals, because two IFN-γ genes seem to exist in some teleosts (6), and type I IFN can be classified into two groups based on their primary protein sequences and biological activities (12). Thus, whereas group I IFNs from phylogenetically distant species were potent at inducing Mx expression and eliciting antiviral responses (10–16), group II IFN from the rainbow trout was poor in these activities (12). In this study, we have demonstrated in the zebrafish that although group II IFNs are unable to promote an inflammatory response or to contribute to the clearance of a bacterial infection, they are able to elicit a rapid and transient expression of antiviral genes, and this leads to the protection of fish against the viral infection. In contrast, group I IFN induces antiviral as well as proinflammatory genes at higher levels, but much more slowly, which might explain its dual antiviral and antibacterial activities. Collectively, these results suggest complementary roles for group I and II IFNs in viral infections, where group II IFNs would play a major role during the early stages of these infections, whereas group I IFNs would be responsible for the viral clearance and the resolution of the infection at later stages.

An important contribution of this study is the unexpected in vivo failure of zfIFNγ to modulate the resistance of zebrafish to bacterial and viral infections. Although two linked IFN-γ-like genes were reported in the zebrafish genome (6), we chose the zfIFNγ1–2 for this study because the zfIFNγ1–1 has no homology with the IFN-γ of higher vertebrates and lacks the conserved IFN-γ superfamily domain (Pfam accession no. PF00714), which strongly suggests that it is not a true IFN-γ ortholog. We found that zfIFNγ was unable to significantly increase the expression of proinflammatory and antiviral genes, which might explain its failure to protect the fish against *S. iniae* and SVCV. Interestingly, however, the coadministration of zfIFNγ and IFN1 results in a more rapid induction of antiviral and proinflammatory genes compared with the administration of IFN1 alone. These results contrast with those obtained in the rainbow trout and the goldfish. In the rainbow trout, IFN-γ has been shown to stimulate the in vitro gene expression of the IFN-γ-inducible protein γIP, MHCIIβ, and STAT1 and to enhance the respiratory burst activity in macrophages (7). Similarly, IFN-γ primes goldfish macrophages and neutrophils for enhanced respiratory burst responses and increases the phagocytic and NO responses of macrophages (8). This reflects the difficulties in reconciling the results obtained in vitro from primary cell cultures or cell lines with those obtained in vivo from animals. In fact, we also found that zfIFNγ is able to increase the expression of MxB and MxC in ZF4 cells and to protect them against SVCV. The development of specific cell markers to T lymphocytes and NK cells, as well as the generation of mutant zebrafish lines devoid of these cell populations, will help us to understand the role played by fish IFN-γ and whether it is indeed an immune IFN (i.e., it is produced by activated T lymphocytes and NK cells) or, alternatively, whether it is also a virus-induced IFN. The inability of zfIFNγ to elicit the expression of proinflammatory genes, including that of NO2, which is strongly induced in mice by IFN-γ (21), would argue against a fully conserved function of fish and mammalian IFN-γ. In fact, zfIFN1 is able to rapidly induce the expression of NO2b, which might contribute to the killing of *S. iniae* by macrophages by means of the production of reactive nitrogen and oxygen intermediates. The involvement of fish macrophages in the elimination of *S. iniae* is thought to be essential because the capsule of this bacterium impairs phagocytic clearance and contributes to virulence (22), and the bacterium is able to multiply within macrophages and has the ability to kill macrophages via the induction of apoptotic processes (19).

In conclusion, we have characterized the biological activities of zebrafish IFNs by using two easy and reproducible infection models. The results suggest complementary roles for group I and II IFNs of fish in viral infections; group II IFNs would control the viral replication during the early stages of infection, whereas group I IFNs would display a more powerful but delayed antiviral action. In addition, group I IFNs show a more pleiotropic activity, as they are also involved in the regulation of the inflammatory response and the resolution of bacterial infections. Finally, IFN-γ lacks the powerful proinflammatory and antiviral activities of its mammalian counterpart. These results give way to future studies that will illuminate the phylogeny of IFNs in vertebrates and many aspects of importance to the aquaculture industry.

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