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The role of gamma interferon in innate immunity in the zebrafish embryo

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SUMMARY

The zebrafish genome contains ten genes that encode class II cytokine-like peptides, of which the two that are related most closely to mammalian interferon gamma (IFN-γ) were named IFN-γ1 and IFN-γ2. Although the zebrafish has become a popular model system to study immune mechanisms, and although interferons are central regulators of immunity, which zebrafish cytokines correspond functionally to mammalian IFN-γ has not been established. We used zebrafish embryos to assay the functions of IFN-γ1 and IFN-γ2, and have identified a subset of zebrafish homologs of the mammalian IFN-responsive genes as IFN-γ targets in the zebrafish embryo: these genes are upregulated in response to raised levels of either IFN-γ1 or IFN-γ2. Infection studies using two different pathogens show that IFN-γ signalling is required for resistance against bacterial infections in the young embryo and that the levels of IFN-γ need to be regulated tightly: raising IFN-γ levels sensitizes fish embryos against bacterial infection. Embryos injected with high doses of *Escherichia coli* are able to clear the bacteria within a day, and the γ-interferons are necessary for this defence reaction. The protective response to *Yersinia ruckeri*, a natural fish pathogen that is lethal at low doses, also requires IFN-γ. As in the induction of target genes, the two interferons act at least partly redundantly. Together with the previously demonstrated type III interferon response, these results show that the counterparts of the mammalian viral and bacterial interferon-dependent defence functions are in place in zebrafish embryos, and suggest that zebrafish IFN-γ1 and IFN-γ2 are functionally equivalent to mammalian IFN-γ.

INTRODUCTION

Over the last decades, a number of vertebrate and invertebrate species have been used as model organisms for the study of immunity. Among the classic examples, studies in amphibians, fish and birds have illuminated the function and evolution of the adaptive immune system, whereas, more recently, experiments in insects, nematodes and plants have contributed to the understanding of innate immunity. As a more recent addition to the repertoire of genetic model organisms, especially for the use of large-scale 'forward' genetic screens, the zebrafish has been employed for the study of disease mechanisms and, in particular, pathogen resistance and innate immunity (for reviews, see Meeker and Trede, 2008; Sullivan and Kim, 2008; Burns and Zon, 2006; Pozos and Ramakrishnan, 2004; Trede et al., 2004; van der Sar et al., 2004; Traver et al., 2003; Yoder et al., 2002).

With its comparatively cheap maintenance, accessibility for experimental manipulation and options for large-scale screens, the zebrafish represents an attractive alternative to mammalian model organisms, both for the study of the evolution of immune defence mechanisms, and for the understanding and analysis of the basic processes underlying immunity in vertebrates.

The adaptive immune system of the zebrafish develops at about 3 weeks of age (reviewed by Trede et al., 2004). This means that within the first three weeks of development the embryo and larva depend solely on innate immune defence mechanisms. Although the embryo is enclosed in a chorion for the first two days of its life, which protects it physically, it is not known whether some pathogens may not be able to penetrate the chorion. At this stage, the embryo is able already to clear experimentally created bacterial infections and can activate typical immune response target genes, suggesting that innate immune signalling and defence pathways must be active. Little is known about the innate defence mechanisms that the zebrafish uses at this, or other, stages.

Clearance of bacteria is achieved mainly by macrophages and neutrophils, which engulf bacterial pathogens shortly after infection, as shown for *Mycobacterium marinum* (Davis et al., 2002), *Escherichia coli*, *Bacillus subtilis* (Herbomel et al., 1999), *Vibrio anguillarum* (O'Toole et al., 2004), *Staphylococcus aureus* (Prajsnar et al., 2008) and *Salmonella typhimurium* (van der Sar et al., 2003; van der Sar et al., 2006). The zebrafish embryo is also able to counteract viral infections, such as spring viremia of carp virus (Sanders et al., 2003; Levraud et al., 2007), snakehead rhabdovirus (Phelan et al., 2005) and nervous necrosis virus (Lu et al., 2008). As in mammals, infection induces acute phase proteins in the zebrafish embryo. During the early phase of infection bacterial pathogens lead to the induction of *il1b* and *tnfa* (Pressley et al., 2005), whereas viral infections induce *tlr3*, *tnfa*, *mxa*, *mxb*, *mxc*, *mxe*, *viperin* (also known as *rsad2*), *iκbα* and *ifn-φ1* (*ifnphi1*) (Phelan et al., 2005; Novoa et al., 2006; Levraud et al., 2007; Lu et al., 2008). The ability of the fish embryo to react to infection with an effective immune defence is both of biological interest and of importance for practical reasons. It means that embryos rather than adults can be used in assays involving pathogens, which is an advantage because embryos can be kept in small volumes apart from the main fish facilities, thereby avoiding the risk of contamination of stocks.

The major components of the immune recognition systems and defence signalling pathways are highly conserved between fish and

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mammals, but significant divergence has been observed as well, both in the way the adaptive immune system creates diversity, and among the innate immune recognition and effector components that are involved in direct interactions with pathogens (Traver et al., 2003; Meijer et al., 2004; Jault et al., 2004; Litman et al., 2001; Yoder et al., 2004; van der Aa et al., 2009; Stein et al., 2007). One set of fast-evolving components that have a central role in immune defence are the interferons and their receptors, of which only a small subset has been studied in the zebrafish.

Interferons, which are classified into three groups (type I, II and III) in mammals, belong to the family of class II cytokines, which mediate immune and inflammatory responses. Apart from the interferons [namely, the single IFN-γ (type II), three IFN-λs (type III), the large IFN-α (type I) expansions, and several others], this family includes the interleukins IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26. Systematic bioinformatic searches and the isolation of cDNAs encoding interferons or interferon-related peptides from various fish species have shown that there is a large number of class II cytokine genes in teleosts. The only clear relationships between fish and mammalian genes are seen in the interleukin group, whereas the interferons are much more divergent (Fig. 1A). None of the predicted or experimentally characterised fish interferons closely resemble the mammalian type I or type III interferons. However, genes have been found in several fish species that are slightly more similar to IFN-γ than to any of the other mammalian interferons, and that have therefore also been named IFN-γ, even though the relationship to mammalian interferons is not supported by high bootstrap values (Stein et al., 2007). Some fish species, including the zebrafish, have two such genes (Igawa et al., 2006; Milev-Milovanovic et al., 2006; Stolte et al., 2008), whereas in other species of fish, only one gene has been found (Zou et al., 2005). The apparent *ifn-γ* homologs are expressed in immune organs such as the spleen and kidney, as well as in specific immune cells like natural killer (NK) and T cells (Milev-Milovanovic et al., 2006; Stolte et al., 2008; Zou et al., 2005). Zou et al. showed that, in cell culture experiments, the rainbow trout (*Oncorhynchus mykiss*) IFN-γ is functional and leads to the activation of the target gene *Ip-10* and enhanced respiratory burst activity in macrophages (Zou et al., 2005).

Finally, fish have one or more interferons that are not related closely to any of the mammalian interferons: these were named IFN-φs (Stein et al., 2007). This group includes an interferon that has been referred to variously as 'zebrafish interferon', 'IFN A/B', 'type I IFN' and 'IFN-λ' (Altmann et al., 2003; Lutfalla et al., 2003; Phelan et al., 2005; Robertsen, 2006; Wang et al., 2006; Levraud et al., 2007), but the phylogenetic distance of this protein from the mammalian type I and type III interferons does not support this unambiguously, and it was therefore renamed IFNφ1 (ZFIN ID: ZDB-GENE-030721-3).

In summary, from the sequence comparison alone, it is not possible to judge which of the fish genes correspond to which mammalian interferon genes, or which of the predicted fish cytokines may fulfil functions similar to those known for mammalian interferons. However, if the zebrafish is to be used as an experimental system for the study of immunity, inflammation and disease, then an understanding of the interferon signalling system is essential, and functional studies are necessary to determine the roles of the interferons.

Functional studies have been carried out in various fish and in cultured fish cell lines. The zebrafish class II cytokine genes whose functions have been analysed in vivo are *ifn-φ1*, *ifn-φ2* (*ifnphi2*) and *ifn-φ3* (*ifnphi3*). *ifn-φ1* is involved in the activation of virus-induced target genes and is necessary for resistance to viral infections in the zebrafish embryo (Levraud et al., 2007); it is also involved in resistance against viral and bacterial infections in adult zebrafish (Lopez-Munoz et al., 2009). *ifn-φ2* and *ifn-φ3* have been

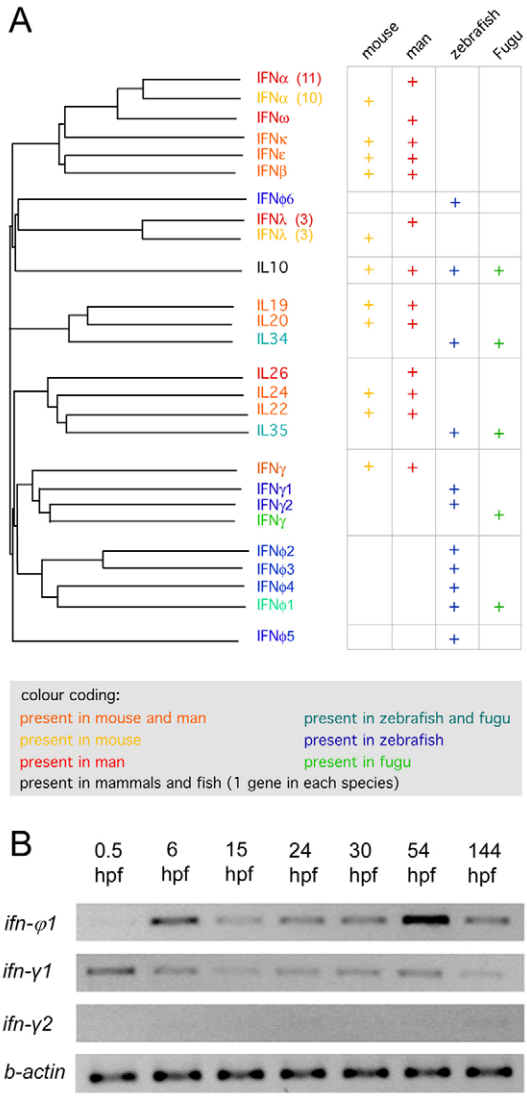

Fig. 1. Interferons in the zebrafish. (A) Phylogram of the class II cytokines showing the different members in the mouse, human, *Takifugu rubripes* (fugu, a species of pufferfish) and zebrafish. This is a highly simplified version of a phylogram from Stein et al. (Stein et al., 2007). Branches with orthologous genes for the mouse and human are collapsed into one branch and marked in orange (e.g. IFN-γ, IL-19, IL-20, IL-22, IL-24, IFN-β, IFN-κ, IFN-ε); branches with orthologous genes for the zebrafish and pufferfish are collapsed into one branch and marked in turquoise (IL-34, IL-35, IFN-φ1), except in the case of IFN-γ/γ2 where the branches are not collapsed owing to the difference in names. Species-specific expansions of the α and λ interferons are also collapsed into one branch. (B) RT-PCR expression analysis of *ifn-φ1*, *ifn-γ1* and *ifn-γ2* expression during zebrafish development. β-*actin* was used as a control. hpf=hours postfertilisation.

shown recently to mediate resistance against viral infections in adult zebrafish (Lopez-Munoz et al., 2009). However, *ifn-φ1* is the only zebrafish interferon for which the corresponding receptor could be identified (Levraud et al., 2007). It was shown to act through a receptor composed of the cytokine receptor family member B1 (CRFB1) and CRFB5 receptor chains. In view of its antiviral activity, and because of its genomic organization, Levraud and colleagues concluded that IFN-φ1 is most similar to the mammalian type III interferon, IFN-λ (Levraud et al., 2007). This further illustrates that the phylogenetic relationships suggested by sequence comparisons are not a reliable basis for deducing functional analogies.

The two *ifn-γ* genes in the zebrafish are inducible by both lipopolysaccharide (LPS) and polyriboinosinic-polyribocytidylic acid (polyIC) in tissue culture cells (Igawa et al., 2006). One of the genes, *ifn-γ2* (also known as *ifng1-2*), has been shown to induce *mxb* and *mxc* in ZF4 cells and mediates resistance against viral infections in these cells (Lopez-Munoz et al., 2009). However, treatment of adult zebrafish with IFN-γ2 failed to mediate resistance against viral and bacterial infections (Lopez-Munoz et al., 2009). To date, the function of *ifn-γ1* (also known as *ifng1-1*) is completely unknown and nothing is known about the expression, induction and function of *ifn-γ1* or *ifn-γ2* in the zebrafish embryo.

To determine whether the two predicted zebrafish *ifn-γ* genes correspond to mammalian gamma interferons in their innate immune functions, we used the zebrafish embryo to test their roles in activating target genes and defending the fish against pathogens.

RESULTS

Expression of *ifn-γ* during zebrafish development

The zebrafish is able to respond to bacterial infections at an early stage of development. To test whether interferon might be involved in the response, we first analysed the stages during development at which the gamma interferon genes *ifn-γ1* and *ifn-γ2* are expressed. Real-time PCR (RT-PCR) shows high levels of *ifn-γ1* mRNA in the eggs immediately after they are laid: the mRNA must be supplied maternally, since zygotic transcription has not started at this point. *ifn-γ1* mRNA continues to be expressed, although at lower levels, throughout embryonic development (Fig. 1B). *ifn-γ2* mRNA was not found during the early stages of development, but was sometimes seen at very low levels at later stages (Fig. 1B).

The expression of interferon in the absence of infection was unexpected. We wanted to determine whether the expression of the gamma interferons was truly constitutive or whether it might, perhaps, be induced by pathogens present in the fish tanks. Thus, we compared *ifn* expression in regularly obtained embryos with expression in germfree embryos (see Methods). We did not detect any differences in expression levels, suggesting that basal *ifn* expression is independent of the presence of microorganisms (data not shown).

IFN-γ target genes in the early zebrafish embryo

The finding that interferons are expressed in the early embryo raises the question of whether the signalling pathway might be active. We used an overexpression approach to test whether *ifn-γ1* or *ifn-γ2* are able to elicit a response. First, we searched for target genes as a readout for IFN-γ signalling. We selected a set of zebrafish homologs of known mammalian IFN-inducible genes and compared their expression in untreated fish embryos with their expression in embryos injected with in vitro transcribed *ifn-γ1* or *ifn-γ2* mRNA. The mRNA was injected into 1–2-cell stage embryos and mRNA from pooled embryos was isolated for RT-PCR at 28 hpf.

The genes we tested were *ifn-φ1*, *nramp*, *adar*, *mx*, *ifi30*, *cathepsin D* (*ctsd*), *p27* (*cdkn1b*), *cr3*, *gp91*, *lmp2* (also known as *psmb9a*) and members of two interferon-dependent immune GTPases, the *gbp* and *irg* families of genes. These potential target genes responded in different ways. The majority showed a high basal expression, which was not influenced by overexpression of *ifn-γ1* or *ifn-γ2* (Fig. 2). Except for some *irg* family members, which are discussed below, the only genes that responded to IFN-γ were *ifn-φ1* and *lmp2*. Both showed low levels of basal expression and were upregulated strongly following injection of *ifn-γ1* or *ifn-γ2* mRNA (Fig. 2).

The immunity-related GTPase (IRG, p47) family in the zebrafish consists of 11 IRGs and three 'quasi GTPases' (Bekpen et al., 2005). Five of the *irg* genes (*irge3*, *irge4*, *irgf1*, *irgf3* and *irgf4*) were induced strongly by both IFN-γs, and two (*irge2* and *irgg1*) were induced weakly (Fig. 2). One of the quasi GTPases (*irgq1*) also showed a weak induction, whereas the other two (*irgq2* and *irgq3*) showed high levels of basal expression that was not altered by *ifn-γ* overexpression (Fig. 2). All of the genes that reacted to interferon

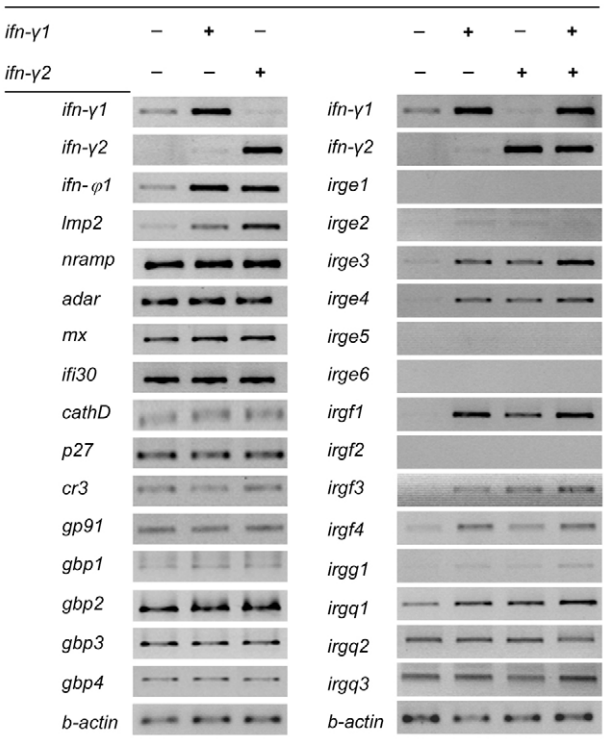

Fig. 2. RT-PCR to identify IFN-γ targets in zebrafish. Expression of the potential target genes was assayed at 28 hpf in untreated control embryos and in embryos injected with *ifn-γ1* and *ifn-γ2* mRNA, as indicated by the '+' signs at the top of the lanes. Injections of mRNA were performed in 1–2-cell stage embryos. To verify successful injections, the levels of *ifn-γ1* and *ifn-γ2* were also tested by RT-PCR. All of the primer pairs used for RT-PCR were able to amplify the predicted products when tested on genomic DNA. β-*actin* was used as a control.

mRNA were induced equally well by *ifn-γ1* and *ifn-γ2*, showing that the two interferons appear to be able to trigger the same responses. In some cases we saw a stronger response to the combination of both interferons, which hints at some non-overlap in their functions, although a particular sensitivity of these genes to the total IFN levels cannot be excluded.

Since both gamma interferons strongly upregulated *ifn-φ1* expression, it was possible that their effect on some of the supposed target genes might, in fact, be indirect, i.e. mediated through the induction of *ifn-φ1*. To test this, we overexpressed *ifn-φ1* and measured the effect on the expression of the postulated gamma interferon targets (*lmp2* and the *irg* genes). As a control for the function of *ifn-φ1* signalling, we tested the expression of the previously identified *ifn-φ1* target gene *viperin* (Levraud et al., 2007). As expected, we found clear *viperin* induction upon *ifn-φ1* overexpression (Fig. 3A). Expression of *ifn-γ1* and *ifn-γ2* was not inducible by *ifn-φ1*, showing that there is no feedback loop from *ifn-φ1* on *ifn-γ* expression (Fig. 3A). The *irg* genes that were not inducible by *ifn-γ* were also not influenced by *ifn-φ1*. Of those genes that responded to *ifn-γ*, only *irge3* and *irge4* were also inducible by *ifn-φ1* (Fig. 3A), whereas *lmp2*, *irgf1*, *irgf3* and *irgf4* were not. Thus, the induction of the *irg* genes in the zebrafish is comparable to the situation in the mouse, where most *irg* genes are specific IFN-γ targets and only a few are inducible by other interferons (Sorace et al., 1995; Bafica et al., 2007; Carlow et al., 1998).

The finding that *ifn-φ1* is able to trigger the expression of *irge3* and *irge4* raises the possibility that the reaction of *irge3* and *irge4* to *ifn-γ* overexpression is mediated through *ifn-φ1*. To test this, we overexpressed *ifn-γ1* and *ifn-γ2* and, in parallel, blocked the *ifn-φ1* signalling pathway using a morpholino (*crfb1*-MO) that was specific for the IFN-φ1 receptor chain CRFB1, which has previously been used successfully (Levraud et al., 2007). In this situation we still observed the activation of *irge3* and *irge4*, but not of the *ifn-φ1* response marker *viperin* (Fig. 3A,B). This shows that *irge3* and *irge4* can be activated by *ifn-γ* independently of *ifn-φ1*. It also shows that the CRFB1 receptor chain is not involved in IFN-γ signalling (Fig. 3B,C).

Since extrinsic RNA can act as a strong immune stimulus, it was possible that activation of the target genes was not the result of IFN-γ production from the injected mRNA, but rather the result of an immune reaction to the injected RNA. To test this, we injected enhanced green fluorescent protein (eGFP) mRNA and monitored the effect on gene expression. eGFP mRNA induced the expression of *ifn-φ1*, *irge3* and *irge4*, whereas the other genes that were upregulated by IFN-γ1 and IFN-γ2 (*irgf1*, *irgf3*, *irgf4* and *lmp2*) did not respond to eGFP mRNA injection (Fig. 3D). Thus, the activation of *ifn-φ1*, *irge3* and *irge4* may be part of an antiviral response, which fits well with the finding that IFN-φ1 is necessary for an antiviral defence (Levraud et al., 2007). By contrast, *irgf1*, *irgf3*, *irgf4* and *lmp2* appear to be true IFN-γ targets.

In conclusion, these data show that the IFN-γ signalling pathway can be activated in the early zebrafish embryo and that all signalling components needed to induce specific IFN-γ target genes must be present.

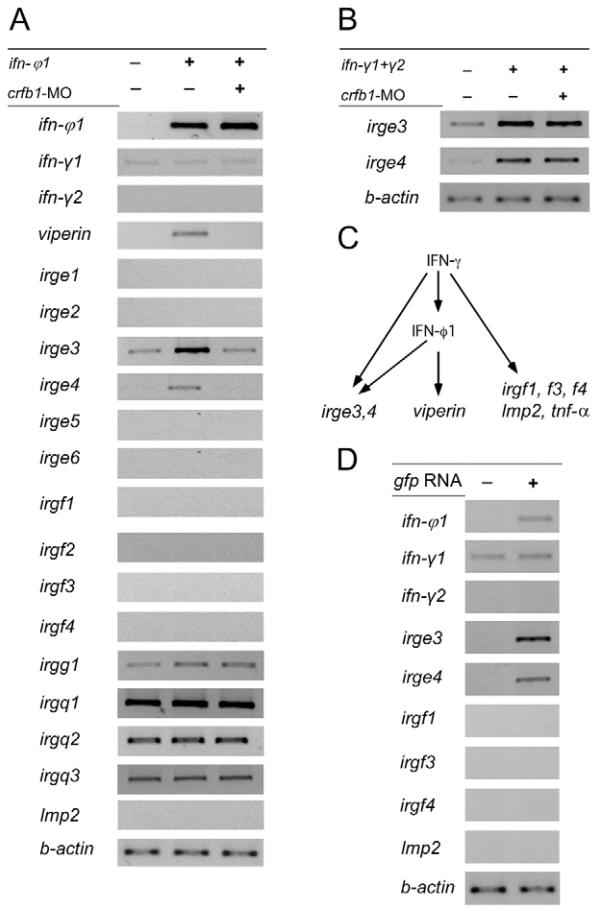

Fig. 3. Influence of IFN-φ1 on IFN-γ target genes. (A) RT-PCR expression analysis to monitor the effects of *ifn-φ1* overexpression on IFN-γ target genes. The expression was assayed at 30 hpf in control (untreated) embryos, *ifn-φ1* RNA-injected embryos and embryos injected with *ifn-φ1* RNA plus a *crfb1*-specific morpholino (*crfb1*-MO). β-*actin* was used as a control. (B) RT-PCR to test the *ifn-φ1* dependence of *irge3* and *irge4* induction by *ifn-γ1* plus *ifn-γ2*. IFN-φ1 signalling was blocked using a morpholino that was specific for the IFN-φ1 receptor chain gene *crfb1*. Expression was tested in control (untreated) embryos, *ifn-γ1* plus *ifn-γ2* RNA-injected embryos, and *ifn-γ1* plus *ifn-γ2* RNA-injected embryos combined with a *crfb1* morpholino injection, as indicated. β-*actin* was used as a control. (C) Scheme showing the hierarchy of IFN-γ, IFN-φ1 and the identified target genes. (D) Response of target genes to control (eGFP) mRNA injection.

Response of interferon genes to bacterial infections

Having found that *ifn-γ* mRNA is present at low levels in the embryo, and that the system necessary to respond to interferon is functional, we wanted to determine whether bacterial infections were able to stimulate interferon signalling. We used two different bacterial strains, *E. coli* (DH5α) and *Yersinia ruckeri*, a natural fish pathogen (for a review, see Tobback et al., 2007), for this purpose. Both bacterial strains carried plasmids that constitutively expressed the red fluorescent protein dsRED so that their presence in the infected fish could be monitored easily (Fig. 4C,D). We initially infected the animals by incubation with bacteria. The bacteria were taken up and were detectable in the intestine, but did not spread from there and did not kill the animals. We therefore turned to injecting bacteria into the posterior blood island at 28 hpf. Embryos survived the injection of doses of up to ~3000 colony-forming units (c.f.u.) of *E. coli* (DH5α), and were able to clear the infection within 2-3 days (Fig. 4A). Thus, we used high doses of *E. coli* (2000-3000 c.f.u.) for

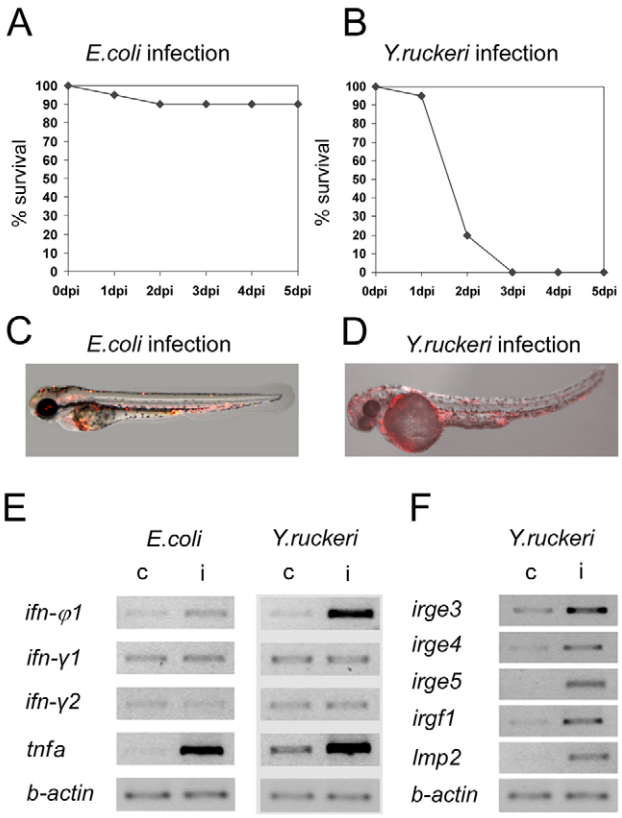

Fig. 4. *E. coli* and *Y. ruckeri* infection of zebrafish embryos. (A) Survival of zebrafish embryos infected with *E. coli* (data represent the mean of two independent experiments; n=40). Embryos were infected with 3000 c.f.u. of *E. coli* at 28-30 hpf and monitored for survival. The percentage of surviving embryos was plotted against days post-infection (dpi). (B) Survival of zebrafish embryos infected with *Y. ruckeri*. Embryos were infected with 2-3 c.f.u. of *Y. ruckeri* at 28-30 hpf to monitor survival (data represent the mean of two independent experiments; n=40). The percentage of surviving embryos was plotted against dpi. (C) An embryo infected with *E. coli* expressing dsRed at 54 hpf (24 hpi). (D) An embryo infected with *Y. ruckeri* expressing dsRed at 48 hpf (18 hpi). (E) Cytokine induction upon *E. coli* and *Y. ruckeri* infection monitored by RT-PCR. Expression was analysed in control embryos (c) and *E. coli*-infected embryos (i, left panel) or *Y. ruckeri*-infected embryos (i, right panel) at 54 hpf (24 hpi). *tnfa* was used to demonstrate an immune response of the embryo. β-*actin* was used as a control. (F) Induction of IFN target genes upon *Y. ruckeri* infection. Expression was tested in control embryos (c) and *Y. ruckeri*- infected embryos (i) at 54 hpf (24 hpi). All putative *ifn-γ* targets were assayed, but only the genes that were upregulated upon infection are shown here. The following genes showed no change in expression levels: *irge1*, *irge2*, *irge6*, *irgf2*, *irgf3*, *irgf4*, *irgg1*, *irgq1*, *irgq2*, *irgq3*. β-*actin* was used as a control.

subsequent experiments. By contrast, the injection of as few as ~15-20 c.f.u. of *Y. ruckeri* killed the embryos within 2 days (Fig. 4B).

The expression of target genes was measured by RT-PCR at 24 hours after infection. To monitor the inflammatory response, we assayed the expression of *tnfa*, which has been shown previously to be upregulated strongly in zebrafish infected with *Edwardsiella tarda* (Pressley et al., 2005).

Both *E. coli* and *Y. ruckeri* induced a strong upregulation of *tnfa* expression showing that the infection was recognised and that innate immune signalling cascades were activated (Fig. 4E). *ifn-φ1* was also upregulated, but much more efficiently by *Y. ruckeri* than by *E. coli*, whereas neither pathogen induced upregulation of *ifn-γ1* or *ifn-γ2* (Fig. 4E), even when tested early after infection [3 and 6 hours post-infection (hpi), not shown].

We also tested whether *Y. ruckeri* infection leads to the activation of the *irg* genes or other *ifn-γ* target genes. Consistent with the finding that *ifn-φ1* is upregulated upon infection, expression of the *ifn-φ1*-inducible *irg* genes *irge3* and *irge4* was also upregulated (Fig. 4F). Unexpectedly, *irge5* transcription was also activated upon *Y. ruckeri* infection, although this gene did not respond to any of the interferons in the overexpression studies, indicating that it must be activated by an alternative pathway or by an as-yet-unidentified member of the interferon family.

Despite the fact that *ifn-γ1* and *ifn-γ2* mRNA levels remained constant after infection, their target genes *irgf1* and *lmp2* were activated upon *Y. ruckeri* infection (Fig. 4F). This cannot be an indirect result of IFN-φ1 being upregulated by *Y. ruckeri*, since *irgf1* and *lmp2* do not respond to IFN-φ1. Thus, bacterial infection either induces these genes through an interferon-independent pathway, or the interferon produced from the existing low levels of mRNA, which is not sufficient to induce target genes on its own, may be sufficient to do so in the presence of signals from a separate pathway triggered by bacterial infection [e.g. Toll-like receptor (TLR) signalling]. Alternatively, the pathway triggered by infection may lead to the post-transcriptional upregulation of interferon production from the low level of mRNA.

Effects of altered *ifn-γ* levels during infection

The fact that interferon signalling is functional in the embryo and responsive to infection suggests that it might have a role in protecting the embryo against pathogens. To test this, we analysed whether raising or lowering the levels of interferons affected the reaction of the embryos to bacterial infection. We either overexpressed *ifn-γ1* and *ifn-γ2* or carried out knockdowns using antisense morpholino oligonucleotides (MO) against the 5′ untranslated region (UTR) of *ifn-γ1* and *ifn-γ2* and compared the effect on infections with that of untreated sibling embryos. To block IFN-φ1 signalling, we used the morpholino directed against the IFN-φ1 receptor chain CRFB1.

To test the efficacy of the morpholinos, GFP constructs were generated that contained the morpholino target sequences upstream of eGFP. In vitro transcribed fusion RNAs (*crfb1*–eGFP, *ifn-γ1*–eGFP and *ifn-γ2*–eGFP) were injected into 1-cell stage embryos with or without the corresponding morpholinos. Injection of the fusion eGFP-RNAs led to strong fluorescence in the injected embryos, which was effectively shut down by the corresponding morpholinos (Fig. 5A). Thus, all three morpholinos blocked translation efficiently.

We assayed the survival rates of embryos that were injected with *crfb1*, *ifn-γ1* and *ifn-γ2* morpholinos and infected with ~3000 c.f.u. of *E. coli*. Embryos in which any one of the signalling pathways had been blocked were able to clear the bacteria and showed survival rates that were comparable to untreated embryos (Fig. 5B), indicating that knockdown of a single interferon signalling pathway does not abolish resistance to *E. coli*. It also shows that the morpholinos that we used had no unspecific negative effects on resistance to bacteria. However, only 24% of embryos injected simultaneously with *ifn-γ1* and *ifn-γ2* morpholinos (*ifn-γ1*+*ifn-γ2*

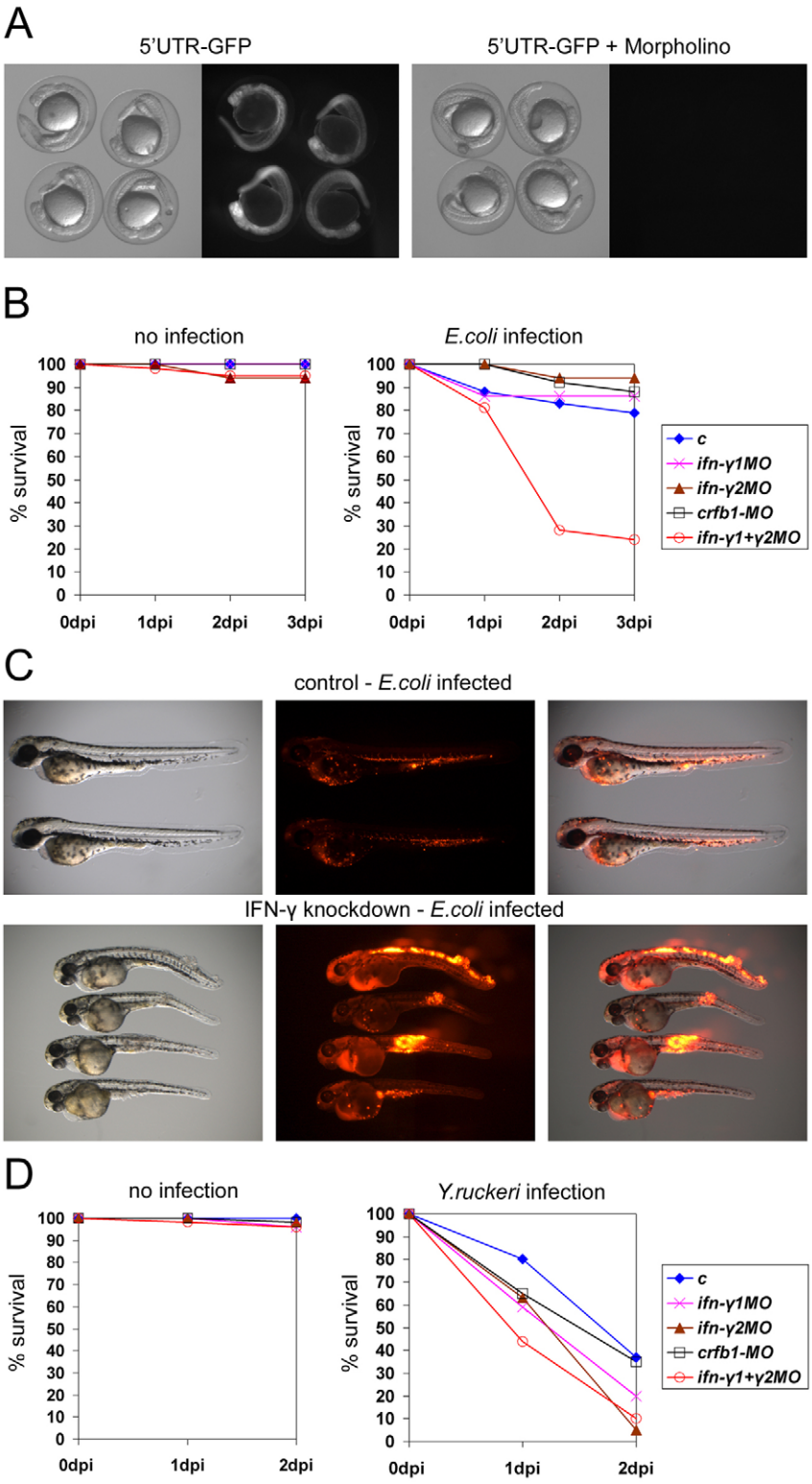

Fig. 5. Survival of IFN knockdown embryos after bacterial infection. (A) Control experiments to verify the morpholino-mediated knockdown. Bright field and eGFP fluorescence images of embryos at 24 hpf. The embryos were injected with *ifn-γ2* 5′UTR-GFP RNA alone (left panels) or together with a morpholino specific for the *ifn-γ2* translation start site (right panels). Similar results were also obtained for the *ifn-γ1*- and *crfb1*-specific morpholinos. (B) Survival of *ifn-γ* knockdown embryos infected with *E. coli*. Embryos were left untreated (control) or injected with morpholinos specific for *ifn-γ1*, *ifn-γ2* or *crfb1* alone, or in combination, as indicated (data represent the mean of two independent experiments; *n*=40 for each treatment). x-axis: percentage of surviving embryos; y-axis: dpi. The left panel shows the survival rates for embryos that were not infected. The right panel shows the survival rates for the embryos that were infected with *E. coli* (3000 c.f.u.) at 28-30 hpf. (C) Embryos infected with dsRed-expressing *E. coli* at 24 hpi (54 hpf). Upper panels: infected control embryos; lower panels: infected *ifn-γ1+ifn-γ2* morpholino-injected embryos. Left panels: bright field; middle panels: dsRed fluorescence; right panels: overlay. Time course images of the infection can be found in the supplementary material (Fig. S2). (D) Embryos were left untreated (control) or injected with morpholinos specific for *ifn-γ1*, *ifn-γ2* or *crfb1* alone, or in combination, as indicated (data represent the mean of two independent experiments; *n*=50 for each treatment). x-axis: percentage of surviving embryos; y-axis: dpi. The left panel shows the survival rates for embryos that were not infected. The right panel shows the survival rates for embryos that were infected with *Y. ruckeri* at 28-30 hpf. The survival of each of the populations of treated embryos was compared to that of the untreated embryos using the log-rank (Mantel-Cox) test. The numbers for day 1 and 2 (*Y. ruckeri*) or days 1 to 3 (*E. coli*) were pooled. The χ^2 values for the comparisons were as follows (χ^2<3.84 is not statistically significant; significant values are underlined): *Y. ruckeri*: CRFB1=0.48, IFNg1=3.36, IFNg2=6.37, IFNg1+2=8.42; *E. coli*: CRFB1=0.46, IFNg1=1.38, IFNg2=2.38, IFNg1+2=15.24.

MO-injected embryos) survived the *E. coli* infection (Fig. 5B). This was also reflected in the extent of bacterial clearance in the surviving embryos at 24 hpi. Whereas the infection was moderate in untreated embryos at 24 hpi (Fig. 5C), the *ifn-γ1+ifn-γ2* MO-injected embryos showed a strong infection that was more widespread (Fig. 5C). The *ifn-γ1+ifn-γ2* MO-injected embryos developed completely normally and did not show any signs of retardation before infection (supplementary material Fig. S1). This is also reflected by the high survival rate of their non-infected siblings (Fig. 5B). In order to further strengthen the conclusion that

the failure in the defence of the embryos against bacteria is the result of interferon downregulation, it would be desirable to rescue the defects in the morpholino-treated embryos by co-injection of a morpholino-insensitive interferon mRNA. This is impossible because the expression of interferon from injected mRNA also increased sensitivity to infection (described in more detail below). However, the observed redundancy, that is, the fact that resistance was lost only when both morpholinos were used together, makes it unlikely that the reduced resistance was the result of unspecific effects of one or both morpholinos.

Knockdown of both *ifn-γ1* and *ifn-γ2* also increased the mortality caused by infection with *Y. ruckeri* (15 c.f.u.). Only 44% of these embryos survived the first 24 hours after infection compared with 80% of untreated embryos (Fig. 5D). In contrast to *E. coli* infections, *Y. ruckeri* infection also led to slightly increased death rates in embryos in which *ifn-γ1*, *ifn-γ2* or *crfb1* had been knocked down individually (~65% survival at 24 hpi) (Fig. 5D). At 48 hpi, the effect of the *ifn-γ* single knockdowns was similar to that seen in embryos with the double knockdown.

These experiments show that IFN-γ1 and IFN-γ2 can protect the early zebrafish embryo against bacterial infections. The finding that either of the gamma interferons is sufficient to protect the embryo against *E. coli* suggests that they have largely or completely overlapping functions, which is consistent with their effects on target gene activation. The fact that the single knockdowns nevertheless had an effect on the defence against *Y. ruckeri* might be because of a quantitative effect – perhaps the amount of protein produced from only one of the interferon genes is simply not sufficient to protect against highly virulent pathogens. To test whether higher levels of IFN-γ can protect the embryo against the lethal effect of *Y. ruckeri* infection, we overexpressed *ifn-γ1* and *ifn-γ2* and assayed the survival of the embryos. However, rather than protection, we observed a higher death rate in the *ifn-γ*-overexpressing embryos. Whereas the infected control embryos showed survival rates of ~95% at 1 dpi, only 15% of the *ifn-γ* RNA-injected embryos survived to this time point when infected with 15 c.f.u. of *Y. ruckeri* (Fig. 6A). A comparable effect on survival after infection has been observed for tumour necrosis factor a (Tnfa). Roca et al. found that zebrafish show a higher susceptibility to viral (spring viremia of carp virus) and bacterial (*Streptococcus iniae*) infection after Tnfa pre-treatment (Roca et al., 2008). Hence, we were interested to see whether the *ifn-γ* overexpression influences *tnfa* expression in the early embryo. When we compared *tnfa* expression in control embryos with *ifn-γ*-overexpressing embryos, we found an upregulation of *tnfa* upon *ifn-γ* overexpression (Fig. 6B). Furthermore, the level of *tnfa* in *ifn-γ*-overexpressing embryos was enhanced further when the embryos were infected with bacteria (Fig. 6B). A similar effect was seen for *il1b* expression, although the induction of *il1b* by *ifn-γ* is weaker than the expression of *tnfa* (Fig. 6B). Thus, the strong activation of *ifn-γ* might lead to a burst of expression of downstream cytokines, which appear to be deleterious for the infected embryo.

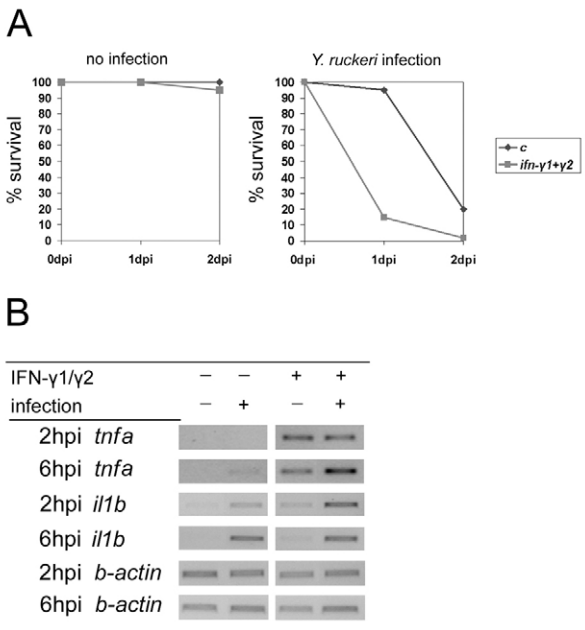

Fig. 6. Effect of *ifn-γ* overexpression on embryo survival after *Y. ruckeri* infection. (A) Left panel: uninjected control embryos (*n*=65) and embryos that were injected with *ifn-γ1+ifn-γ2* mRNAs (*n*=59) but not infected with bacteria. Right panel: infection of control embryos (*n*=65) and *ifn-γ1+ifn-γ2* RNA-injected embryos (*n*=59) with *Y. ruckeri* (15 c.f.u.) at 28-30 hpf. (B) Induction of *tnfa* and *il1b* by *ifn-γ* and bacterial infection. Expression was compared in control embryos, infected control embryos, *ifn-γ1+γ2* RNA-injected embryos and *ifn-γ1+γ2* RNA-injected embryos with an additional infection. Infections were performed at 28-30 hpf. Expression was assayed 2 and 6 hpi.

DISCUSSION

We have found that the IFN-γ signalling system can be activated in the early zebrafish embryo and is able to upregulate several target genes. Many of the homologs of known mammalian gamma interferon-inducible genes that we tested showed high levels of constitutive expression and did not respond to IFN-γ (*nramp*, *adar*, *mx*, *ifi30*, *cathepsinD*, *p27*, *cr3*, *gp91*, *gbp1-4*). At least at this early stage, these genes must therefore be subject to a different mode of regulation. Their constitutive expression is surprising because, in mammals, most of them show no detectable expression in the absence of interferon, and the expression of immune genes often comes with a cost to the organism.

It is worth speculating about the biological relevance of the constitutive expression of innate immune defence genes in the early zebrafish embryo. A role in immune defence should not be needed during the first two days because the embryo is enclosed in a chorion that protects it from pathogens. We consider it unlikely that these genes have an alternative function in the early embryo. One possibility, a developmental function, is unlikely since developmental mechanisms in vertebrates are highly conserved and no such roles have been described for these genes in other species. However, as soon as the embryo hatches, it is immediately exposed to the pathogens of the environment, so perhaps the pre-existence of certain components of the defence system is of advantage at this point.

The *irg* genes

In contrast to the immune GTPases of the guanylate binding protein (GBP) and myxovirus resistance (MX) type, which are expressed constitutively in the zebrafish embryo, some members of the p47 IRG family show different behaviours. The three *irgq* genes (*irgq1-3*) are also expressed constitutively, whereas the members of the

irge and *irgf* group are not (or only minimally) expressed in untreated fish embryos. Based on the absence of a functional nucleotide-binding motif, it has previously been suggested that the function of the Irgq group members has diverged from that of the other IRGs (hence the name q or quasi GTPases) (Bekpen et al., 2005). The difference in their regulation in the zebrafish embryo supports this notion.

irge3, *irge4*, *irgf1*, *irgf3* and *irgf4* were induced strongly by interferon; *irge2* was activated weakly. Since no cDNAs had been reported previously for *irgf4* and *irge2*, this is the first experimental support for the prediction of these two genes. *irgg1* is activated very weakly by interferon; this gene lies in tandem with the constitutively expressed *irgq1*, which showed some activation above its basal level upon interferon stimulation. These two genes have in fact been annotated as a fusion gene where the sequences encoding the *irgg1* exon are spliced to the downstream *irgq1* exon. The different expression and regulation of the sequences amplified by our two exon-specific primer sets indicates that independent transcripts must be made. Furthermore, RT-PCR using primer pairs that should amplify transcripts containing both *irgg1* and *irgq1* did not yield any such products, suggesting that there are two independent genes. Nevertheless, it is possible that the response we see for *irgq1* after interferon treatment may be because of the promoter activity of the upstream *irgg1* gene.

The remaining IRG family members, *irge1*, *irge5*, *irge6* and *irgf2* are not expressed in either the absence or presence of interferon. For *irgf2*, this finding is consistent with the fact that no expressed sequence tags (ESTs) have been found. An EST does exist for *irge1* and we also detect expression in adult tissues (unpublished data) showing that it can be expressed, but apparently it does not respond to interferon.

The genes of the *irge* and *irgf* groups are the strongest interferon responders that we have found in the zebrafish embryo. This was possibly unexpected for the members of the *irgf* group. The zebrafish *irgf* genes resemble the '*irgc-like*' genes in mammals in that they have an intron in the same place in the middle of the main exon that encodes the G-domain (Bekpen et al., 2005) (Christoph Rohde and Jonathan C. Howard, unpublished results), and because the mammalian genes in this class are not inducible by interferon.

Redundancy of *ifn-γ1* and *ifn-γ2*

The zebrafish genome and many other teleost genomes contain two *ifn-γ* genes that show only a low degree of identity to each other (18.8% amino acid identity between zebrafish *ifn-γ1* and *ifn-γ2*) (Zou et al., 2005; Igawa et al., 2006; Milev-Milovanovic et al., 2006; Stolte et al., 2008). Our results indicate that *ifn-γ1* and *ifn-γ2* act redundantly despite their low degree of sequence similarity. We did not find any target genes that were inducible by one interferon and not the other, and knockdown of either interferon alone did not interfere with the ability of the embryos to clear *E. coli* that were injected into the posterior blood island.

One finding that might argue against a complete functional equivalence of *ifn-γ1* and *ifn-γ2* is the increased mortality of *Y. ruckeri*-infected embryos when expression of only one of the two interferons is blocked. However, this may simply be the result of halving the total interferon levels. Clearly, the embryos are more sensitive to *Y. ruckeri* (it is lethal even at low doses) than to *E. coli*. Thus, the reduction in overall interferon levels might lead to a more severe loss in resistance against *Y. ruckeri* than against *E. coli*. The functional equivalence of the two interferons in the response to *Y. ruckeri* is also consistent with the finding that knockdown of each interferon results in a similar reduction in survival rates, showing that, in each case, the remaining interferon is equally effective at inducing a low-level defence.

One detail in which the two zebrafish interferon sequences differ from each other is that IFN-γ2 contains a nuclear localisation signal (NLS) in the same position as in mouse and human IFN-γ, whereas IFN-γ1 does not. Nuclear localisation of mammalian IFN-γ by its NLS has been proposed to be necessary for full biological activity (Subramaniam et al., 2000), although a mechanism by which IFN crosses the plasma membrane to reach the cytosol has not been described. Our finding that IFN-γ1, without an NLS, is able to trigger a signalling cascade as efficiently as the NLS-containing IFN-γ2 indicates that an NLS is not a general requirement for IFN-γ signalling.

Overexpression of *ifn-γ1* and *ifn-γ2* in the embryo leads to a dramatically increased susceptibility to bacterial infection [a recent report suggests that this reaction is less pronounced in adult fish (Lopez-Munoz et al., 2009)], in a similar way to that shown to occur in mice (Kohler et al., 1993). The increased sensitivity to *E. coli* in mice treated with IFN-γ has been ascribed to a septic response. Sepsis is a dysregulation of inflammation in which an initial systemic inflammatory response, which includes the upregulation of inflammatory cytokines such as TNF-α and IL-1, is not balanced properly by a compensatory anti-inflammatory response (reviewed by Buras et al., 2005). It is possible that the effects we observe also resemble sepsis. We find that *ifn-γ* does indeed induce the expression of the *tnfa* and *il1b* genes, which are upregulated further upon infection, indicating an overactivation of the systemic inflammatory response. This overactivation may also be the cause of the high mortality effects that have been described in adult zebrafish treated with Tnfa, which show enhanced susceptibility to viral and bacterial infection (Roca et al., 2008). However, in contrast to the situation in mammals, injection of high numbers of *E. coli* alone does not induce a septic shock, and fish show a high tolerance to LPS (Novoa et al., 2009). This may be related to the fact that fish recognise bacterial surface components, such as LPS, in a different manner from mammals (Sepulcre et al., 2009). The extent to which a septic response in fish is related to sepsis in mammals will need to be investigated in the future.

METHODS

Morpholino oligonucleotide design, overexpression constructs and injections

Morpholino oligonucleotides (MO) were synthesised by Gene Tools and injected into 1–2-cell stage zebrafish embryos. The injection solution consisted of 0.6 mM of the respective MO, 0.2% Phenol Red and 0.1 M KCl of which, on average, 2 nl were injected per embryo. Injections were carried out using an Eppendorf FemtoJet and micromanipulator.

The MO sequences used were: *ifn-γ1*, 5′-TTTCTGTGCTGT-GAACCAAGTGATG-3′; *ifn-γ2*, 5′-TGAAGGCGTTCGC-TAAAGTTAGAGT-3′; *crfb1*, 5′-GAGTCACACTTTAGCAAT-GATGAAG-3′.

To test the binding of the different MOs, the 5′ sequences of the respective genes were amplified with primers that contained restriction sites for *Eco*RI and *Nco*I or *Bam*HI and *Eco*RI (ifn-γ1-5′utrecofor, 5′-AGGAATTCCCTTTGCCTATATAAACCCC-3′; ifn-γ1-5′utrncorev, 5′-ATCCATGGTTGTGTTTCTGTGCTGTGAA-3′; ifn-γ2-5′utrecofor, 5′-AGGAATTCTCACTCGAACACATGCATTC-3′; ifn-γ2-5′utrncorev, 5′-ATCCATGGAGTTGAAGGCGTTCGCTAAA-3′; crfb1-5′utrbamfor, 5′-GACTGGATCCGAACCGGGTGTAGTGACGTT-3′; crfb1-5′utrecorev, 5′-CTAGGAATTCCACAAACAAAAGCAGAGTCACA-3′). Digested and purified PCR products were cloned into the pCS2+/eGFP vector (kindly provided by Alexander Reugels). Capped RNA was synthesised in vitro using the Message Machine kit (Ambion), according to the supplier's manual. RNA was injected at 200 ng/µl, with and without the respective MO (0.6 mM), into 1–2-cell stage embryos. On average, the injected volume was 2 nl. GFP fluorescence was visualised at 28-32 hpf using a Leica MZ 16FA stereomicroscope.

For overexpression of *ifn-γ1*, *ifn-γ2* and *ifn-φ1*, the coding regions were amplified with primers that contained restriction sites for *Xho*I and *Xba*I (ifn-γ1XhoI-for, 5′-ATCTCGAGATGGATTCCTGCCTCAAAAT-3′; ifn-γ1XbaI-rev, 5′-ATTCTAGACTAAGACATGTGTAAATGCCG-3′; ifn-γ2XhoI-for, 5′-GCCTCGAGATGATTGCGCAACACATGAT-3′; ifn-γ2XbaI-rev, 5′-CGTCTAGATCAACCTCTATTTAGACT-3′; ifn-φ1XhoI-for, 5′-CGCTCGAGATGTGGACCTATATTTTTGTG-3′; ifn-φ1XbaI-rev, 5′-ATTCTAGATCAAGGATTGACCCTTGCGT-3′). The PCR product was gel purified, digested and cloned into the pCS2+ vector (Turner and Weintraub, 1994). For overexpression, we either injected plasmids at a concentration of 100 ng/µl or RNA (200 ng/µl), as described above, into 1–2-cell stage embryos.

RNA isolation and RT-PCR

RNA from zebrafish embryos was isolated from regularly kept embryos or from germfree embryos. To remove germs, the embryos were bleached immediately after fertilization. Bleaching was performed by two cycles of 4-minute incubations in 0.012% sodium hypochlorite followed by two 3-minute washes in sterile 1/3 zebrafish Ringer solution. To test the efficacy of bleaching, we homogenised embryos and plated them on bacteriological plates (LB medium and Middlebrook 7H10). The Middlebrook 7H10 and LB medium plates were incubated at 28°C and 37°C for 7 days. No colonies were detected for bleached embryos, whereas plates with untreated embryos contained ~1000 colonies at 28°C and 37°C. RNA from zebrafish embryos was isolated using the µMacs mRNA isolation kit (Miltenyi), according to protocol, by addition of a DNAseI digest for 7 minutes to remove traces of genomic DNA. First-strand synthesis was performed by utilizing the Superscript III system (Invitrogen) using 300 ng of mRNA and random hexamer primers. For RT-PCR, 7.5 ng first-strand synthesis (7.5 ng) and RedTaq DNA polymerase (Sigma-Aldrich) were used. RT-PCR was performed as follows: 2 minutes at 94°C; 28-35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 1 minute; 7 minutes at 72°C. See supplementary material Table S1 for the primer sequences of the tested genes. All of the primer pairs that were used for RT-PCR were able to amplify the predicted products when tested on genomic DNA. The PCR products were controlled by agarose gel electrophoresis.

TRANSLATIONAL IMPACT

Clinical issue

Innate immunity is an organism's first line of defence against infectious pathogens. Bacterial infection induces the production of cytokines, such as interferon gamma (IFN-γ), which activate non-specific immune-mediating cells, such as macrophages and neutrophils. This type of immune response is primarily responsible for the clearance of bacteria after acute infection. However, the mammalian immune system and inflammatory response are very complex, so a simplified model for bacterial infection may allow more direct investigation of the innate immune response.

The zebrafish is quickly becoming a preferred model for many human diseases, as it is easy to manipulate and alter genetically. The zebrafish embryo does not have an adaptive immune system, which suggests that it may serve as a simple system to study pathogen-induced innate immune mechanisms. However, limited knowledge about zebrafish immune function prevents full realisation of its potential utility as a model for the mammalian immune system.

Results

This work identifies an important signalling system involved in pathogen resistance in the zebrafish embryo, which is mediated by IFN-γ. The authors show that two interferons, IFN-γ1 and IFN-γ2, exhibit similar functions in zebrafish as in mammals, in spite of their sequence divergence. Both IFN-γ types are active in the early zebrafish embryo and are necessary for resistance to pathogens. This work establishes infection of the zebrafish by the Gram-negative bacterium *Yersinia ruckeri* as a tractable experimental tool to define the innate immune response to bacterial infection.

Implications and future directions

Since the function of IFN-γ signalling is conserved in the zebrafish embryo, where it is necessary for survival of the organism following bacterial infection, it should be a useful model to understand the innate immune response to infection. The zebrafish model is easily handled, maintained, observed and manipulated, both experimentally and genetically. Thus, the zebrafish embryo has potential to further define IFN-γ signalling in genetic studies of relevant immunological pathways and for screening drugs that modify these pathways.

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Bacterial strains, media and infections

E. coli (DH5α) and *Y. ruckeri* (strain 4015726Q) carrying the dsRED-expressing pGEMDs3 plasmid (van der Sar et al., 2003) were grown in standard LB medium containing ampicillin (50 µg/ml). For infections, a 3 ml overnight culture was centrifuged for 3 minutes at 4000 ***g***, the supernatant was removed, and the pellet washed with PBS. Washing was repeated three times to remove traces of LB and metabolites. After washing, the pellet was resuspended in 500 µl PBS. The final injection solution contained the washed *E. coli* or *Y. ruckeri* diluted in PBS containing 1/10 Phenol Red.

Zebrafish were bred at 26.5°C on a 14-hour light/10-hour dark cycle. Embryos were collected by natural spawning and staged according to Kimmel et al. (Kimmel et al., 1995).

At 28-30 hpf, zebrafish embryos were anesthetised using tricaine (Sigma-Aldrich), according to standard protocols (Westerfield, 1995), and arranged on agarose plates for infection. Infection was achieved by injecting the bacteria into the embryonic blood island. To determine the amount of injected bacteria, a control injection was performed in 100 µl of sterile PBS and plated on LB-Amp plates to count the colonies. After infection, embryos were washed in 1/3 zebrafish Ringer solution (Westerfield, 1995) and incubated further

at 28.5°C in 1/3 zebrafish Ringer solution to score survival or for RNA isolation. The infection in untreated and *ifn-γ* knockdown embryos was monitored by visualizing dsRed fluorescence using a Leica MZ 16FA stereomicroscope.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

D.S. and M.L. conceived and designed the experiments; D.S., C.S. and D.N. performed the experiments; A.M.v.d.S. constructed fluorescent bacteria; D.S., C.S. and M.L. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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