Prominent amphibian (*Xenopus laevis*) tadpole type III interferon response to the Frog Virus 3 ranavirus

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Running Head: *X. laevis* tadpoles anti-FV3 IFNλ response

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ABSTRACT

Ranaviruses (*Iridoviridae*) are posing an increasing threat to amphibian populations, with anuran tadpoles being particularly susceptible to these viral infections. Moreover, amphibians are the most basal phylogenetic class of vertebrates known to possess both type I and type III interferon-mediated immunity. Moreover, little is known regarding the respective roles of these mediators during amphibian antiviral defenses. Accordingly, we transcriptionally and functionally compared the amphibian *Xenopus laevis* type I (IFN) and III (IFNλ) IFNs in the context of infections by the ranavirus Frog Virus 3 (FV3). *X. laevis* IFN and IFNλ displayed distinct tissue expression profiles. In contrast to our previous findings that *X. laevis* tadpoles exhibit delayed and modest type I IFN responses to FV3 infections compared to adults, here we report that tadpoles mount timely and robust type III IFN gene responses. Recombinant forms of these cytokines (r*Xl*IFN, r*Xl*IFNλ) elicited antiviral gene expression in the kidney-derived A6 cell line as well as in tadpole leukocytes and tissues. However, in comparison to r*Xl*IFN, r*Xl*IFNλ was less effective in preventing FV3 replication in A6 cells and tadpoles, and inferior at promoting tadpole survival. Intriguingly, FV3 impaired the A6 cell and tadpole kidney type III IFN receptor gene expression. Furthermore, compared to r*Xl*IFN, r*Xl*IFNλ conferred equal or greater protection of A6 cultures against recombinant viruses deficient for the putative immune evasion genes, vCARD or a truncated vIF2-α. Thus, in contrast to previous beliefs, tadpoles possess intact antiviral defenses reliant on type III IFNs, which are overcome by FV3 pathogens.

46 **IMPORTANCE**

47 Anuran tadpoles, including those of *Xenopus laevis* are particularly susceptible to infection by
48 ranavirus such as FV3. We investigated the respective roles of *X. laevis* type I and type III
49 interferons (IFN and IFNλ, respectively) during FV3 infections. Notably, tadpoles mounted
50 timely and more robust IFNλ gene expression responses to FV3 than adults, contrasting with the
51 poorer tadpole type I IFN responses. However, a recombinant *X. laevis* (r*Xl*) IFNλ conferred less
52 protection to tadpoles and the A6 cell line than r*Xl*IFN, which may be explained by the FV3
53 impairment of IFNλ receptor gene expression. The importance of IFNλ in tadpole anti-FV3
54 defenses is underlined by the critical involvement of two putative immune-evasion genes in FV3
55 resistance to IFN and IFNλ-mediated responses. These findings challenge the view that tadpoles
56 have defective antiviral immunity and rather suggest that their antiviral responses are
57 predominated by IFNλ responses, which are overcome by FV3.

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INTRODUCTION

Vertebrate antiviral immunity relies heavily in the interferon (IFN) response, which in mammals is comprised of three classes of cytokines, type I, II and III IFNs [1]. IFNγ, the only mammalian type II IFN (bony fish possess multiple type II IFNs [2]) confers a plethora of immune and antiviral roles, whereas type I and III IFNs function predominantly as antiviral molecules. While type I IFNs affect a broad range of cell types, the type III IFNs (also known as IFNλ or IL-28 and IL-29) act on a limited range of cell subsets [3, 4]. These differences are dictated at the receptor level, where the type I IFN receptors; IFNAR1 and IFNAR2 are ubiquitously expressed [5]. By contrast, the type III receptor complex consists of the ligand-binding and IFNλ-specific IFNLR1 chain, which is expressed on a select subset of cells (chiefly amongst these epithelia [6]) and the cell-signal propagating IL10R2 chain (shared with IL-10, IL-22 and IL-26) [7, 8]. Despite these differences, both type I and type III IFN cytokines utilize the same downstream signaling pathways, culminating in comparable antiviral outcomes including increased gene expression of antiviral cellular mediators such as protein kinase R (PKR) and Myxovirus resistance (Mx) proteins [1].

While the mammalian IFN responses have been relatively well characterized, the IFN immunity of phylogenetically more ancestral ectothermic vertebrate species appears to be distinct. At present, only the type I IFN systems of bony fish have been explored in detail, and it is thought that teleosts do not possess type III IFNs. The fish type I IFNs are subdivided into four groups (IFNa-d) according to phylogeny [9, 10], and unlike the single cognate type I IFN receptor complex of mammals [11, 12]; fish group I and II IFNs signal through distinct receptor complexes [13]. We have recently demonstrated that the amphibian *Xenopus laevis* type I IFN is

92 a potent antiviral mediator, conferring considerable protection against the emerging ranaviral
93 pathogen, Frog Virus 3 (FV3, [14]).

94 The mammalian type III IFNs (including interferon lambda; IFNλ-1, -2 and -3; also
95 designated as IL-28A, IL-28B and IL-29) are encoded by five exon/four intron gene transcripts
96 reminiscent of the fish type I IFNs. Intriguingly, although *bona fide* type III IFNs either do not
97 exist, or have not yet been identified in bony fish, amphibians possess both type I IFNs with the
98 five exon/four intron gene organization of their fish counterparts, as well as true type III IFNs
99 [15]. There have been considerable debates regarding the precise phylogenetic relationships of
100 the teleost type I IFNs to the higher vertebrate type I and III cytokines. As such, given their key
101 phylogenetic position as intermediate between fish and mammals, together with their possession
102 of "fish like" type I and "mammalian like" type III IFN genes [15], amphibians are particularly
103 interesting for studying the evolution of antiviral immunity [10, 16, 17].

104 Aside from its inherent fundamental value, a greater understanding of amphibian antiviral
105 IFN defenses is important in the context of emerging infectious diseases caused by ranavirus
106 pathogens (family *Iridoviridae*), which are decimating amphibian populations worldwide.
107 Indeed, the worldwide decline in nearly one-third (32%) of all amphibian species represents an
108 imminent threat to the extinction of these organisms [18]. Moreover, while these die-offs may be
109 attributed to a range of underlying causes [19, 20], the dramatic increase in ranavirus infections
110 and the resulting mortalities suggest that these pathogens are a significant contributing force
111 behind these amphibian declines [18-20]. Ranaviruses are large, icosahedral, dsDNA viruses that
112 manifest in systemic diseases, hemorrhaging and necrotic cell death within multiple afflicted
113 organs [18]. Typically, amphibian tadpoles are more susceptible to, and succumb from these
114 infections, whereas mature adults are usually more resistant to these pathogens [14, 21-24]. Frog

Virus 3 (FV3) is the type species of the ranavirus genus, wherein FV3 infection of the amphibian *Xenopus laevis* presents a pertinent research platform for the study of ranavirus-amphibian immune host interfaces.

Most notably, considering that the frog kidney epithelia is believed to be a primary site of ranaviral replication [25] while the mammalian type III IFNs specifically target epithelial cells [6], insights the question of the roles of the functionally uncharacterized amphibian type III IFNs in the context of anti-ranaviral immunity. Accordingly, we utilized the *X. laevis*-FV3 infection model to address the roles of frog type III IFNs in antiviral immunity.

MATERIALS AND METHODS

Animals. Outbred pre-metamorphic (developmental stage 54; Nieuwkoop and Faber (1956) tadpoles, metamorphic (stage 64) and adult (2 years old) frogs were obtained from our *X. laevis* research resource for immunology at the University of Rochester (http://www.urmc.rochester.edu/mbi/resources/xenopus-laevis/). All animals were handled under strict laboratory and UCAR regulations (Approval number 100577/2003-151).

Identification of *X. laevis* Type III IFN. The *X. laevis* IFNλ cDNA corresponding to the open reading frame was cloned using primers (Table 1) against the *X. tropicalis* IFNλ (Acc. Nb.: KP325221). Briefly, the full-length *X. laevis* IFNλ was amplified by RT-PCR using cDNA derived from FV3-infected *X. laevis* adult spleen as template. The resulting amplicon was cloned into the pGEM-T sequencing vector (Promega), and five individual clones were sequenced.

Frog Virus 3 Stocks and Animal Infections. Fathead minnow cells (FHM; American Type Culture Collection, ATCC No.CCL-42) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/mL) and streptomycin (100 µg/mL)

138 at 30°C with 5% CO_2. FV3 was grown by a single passage in FMH cells and purified via
139 ultracentrifugation on a 30% sucrose cushion. Tadpole kidneys and A6 cells to be assessed for
140 FV3 loads by plaque assays were subjected to 3 rounds of sequential freeze-thaw lysis and
141 repeated passages through a 24-gage needle. All plaque assays were performed on BHK
142 monolayers under an overlay of 1% methylcellulose, as previously described [26].

143 The production and characterization of recombinant FV3 bearing site-specific deletions of
144 18K (82R) and vIF-2 has been previously described [27], while the characterization of ΔvCARD
145 FV3 (open reading frame 64R; 75 529-75 816) is presently in review as a separate manuscript.
146 The two recombinant FV3 were generated by homologous recombination; target genes (FV3
147 genomic location for 52L: 57,481-58,548 and 64R: 75,529-75,816) were PCR amplified from the
148 FV3 genome and cloned into right (restriction sites XhoI and ClaI) and left (restriction sites SacI
149 and SpeI) sides of cassettes bearing a puromycin-resistance gene fused with the coding sequence
150 of EGFP under the control of FV3 immediate-early (IE) gene 18K promoter (18Kprom-Puro-
151 EGFP cassette). Both recombinants were shown to have similar growth kinetics to wild type
152 (WT) virus when cultured in BHK cells, and both have been confirmed to being of high purity by
153 monitoring fluorescence signal in plaque assays and by diagnostic PCR.

154 All tadpole infections were achieved by intraperitoneal (ip) injection of $1x10^4$ FV3 plaque
155 forming units (PFU) in 10 µl volumes. All adult frog infections were performed ip with $5x10^6$
156 FV3 PFU in 100 µl volumes. At indicated times, animals were euthanized by immersion in 0.5%
157 tricaine methane sulfonate (MS-222), tissues and cells were removed and processed for RNA and
158 DNA isolation and plaque forming unit analysis to determine respective FV3 loads.
159 **Quantitative-PCR Gene Expression Analysis.** Total RNA and DNA were extracted from frog
160 tissues and cells using the Trizol reagent following the manufacturer's directions (Invitrogen).

All cDNA synthesis was performed using the iScript cDNA synthesis kit according to manufacturers' directions (Bio-Rad, Hercules, CA) using 500 ng of total DNase treated (Ambion) RNA. Quantitative (q) PCR analysis was performed using 2.5 µl of cDNA templates and 50 ng of DNA templates.

Relative qPCR gene expression analysis of IFN, IFNλ, Mx1, Mx2, PKR, IFNLR1 and IL10R2 were performed via the delta^delta CT method, with expression examined relative to the GAPDH endogenous control and normalized against the lowest observed expression. To measure FV3 viral loads, absolute qRT-PCR was performed on DNA using a serially diluted standard curve. Briefly, an FV3 vDNA Pol II PCR fragment was cloned into the pGEM-T vector (Promega), amplified in bacteria, quantified and serially diluted to yield 10^{10}-10^{1} vDNA POl II fragment-containing plasmid copies. These dilutions were employed as the standard curve in subsequent absolute qPCR assays of FV3 DNA quantities. All experiments were performed using the ABI 7300 real-time PCR system and PerfeCTa® SYBR Green FastMix, ROX (Quanta). ABI sequence detection system software (SDS) was employed for all expression analysis. All primers were validated prior to use.(Table 1).

Generation of r*Xl*IFN and r*Xl*IFNλ insect expression constructs. The production of the *X. laevis* recombinant IFN (r*Xl*IFN) has been previously described [14] and the r*Xl*IFNλ was generated in the same manner. Briefly, full length *X. laevis* IFN and IFNλ sequences without the signal peptide were PCR amplified from FV3-infected adult *X. laevis* spleen cDNA using the iProof high-fidelity DNA polymerase (BioRad) and primers containing HindIII and XhoI restriction sites, designed to meet the requirements of the pMIB/V5 His A insect expression vector (Invitrogen). PCR products were double digested with HindIII and XhoI and ligated into

183 the pMIB/V5 His A. In-frame insertions of *X. laevis* IFN and IFNλ were confirmed by
184 sequencing from both directions.

185 **Production of r*Xl*IFN and r*Xl*IFNλ.** The expression plasmids were transfected into Sf9
186 insect cells using lipofectamine (Invitrogen) and their expression was confirmed by RT-PCR and
187 Western blot using the V5 epitopes. Sf9 insect cells transfected with the r*Xl*IFN- and r*Xl*IFNλ-
188 pMIB/V5 His A were selected using 10 μg/mL blasticidin, scaled up into 500 mL liquid cultures
189 and grown for 5 days under blasticidin selection. Culture supernatants were dialyzed overnight at
190 4°C (150 mM sodium phosphate), concentrated against polyethylene glycol flakes (8 kDa) and
191 dialyzed again. Recombinant proteins were purified by Ni-NTA agarose chromatography
192 (Qiagen). Bound proteins were washed at high stringency (20 volumes of 0.5% Tween 20; 50
193 mM sodium phosphate; 500 mM sodium chloride; 100 mM imidazole), followed by low
194 stringency (5 volumes of 0.5% Tween 20; 50 mM sodium phosphate; 500 mM sodium chloride;
195 100 mM imidazole), and then eluted with 250 mM imidazole. Purity was determined by SDS-
196 PAGE and western blot using the V5 epitope. Protein concentration was determined by the
197 Bradford Protein Assay (BioRad). Protein preparations were aliquoted and stored at 4°C in
198 presence of a protease inhibitor cocktail (Roche).

199 The vector control samples were obtained by transfecting Sf9 cells with an empty expression
200 vector and following the same cell culture and protein purification steps..

201 **Cell Culture Medium.** The ASF culture medium used in these studies has been previously
202 described [28]. All cell cultures were established using ASF supplemented with 10% fetal bovine
203 serum, 20 μg/mL kanamycin and 100 U/mL penicillin / 100 μg/mL streptomycin (Gibco).
204 Amphibian PBS (APBS) has been previously described [28].

A6 cell stimulation and infection. A6 cells ($5x10^5$ per well of 48 well plates), incubated for 6 hrs with 100 ng/mL of either r*Xl*IFN, r*Xl*IFNλ or equal volumes of vector control, were infected with 0.5 MOI of FV3 for an additional 16 hrs. Then RNA and DNA were isolated and cDNA synthesized. To assess dose-dependent effects of r*Xl*IFN and r*Xl*IFNλ, $5x10^5$ A6 cells were treated with 0.5, 5, 50, 500 or 5000 ng/mL of either recombinant cytokine for 6 hrs, infected with 0.5 MOI of FV3 for 16 hrs and harvested for plaque assays.

Tadpole cytokine stimulation and FV3 infections. For tadpole gene expression analysis, tadpoles were injected ip with 1µg of r*Xl*IFN, 1µg of r*Xl*IFNλ or equal volumes of vector control. The following day, tadpoles were euthanized in 0.5% tricaine methane sulfonate (MS-222), cells and tissues isolated and processed for RNA.

For short-term protection assays, stage 54 tadpoles (4/treatment group, *N*=4) were injected ip with 1µg of r*Xl*IFN, 1µg of r*Xl*IFNλ or equal volumes of the vector control and six hours later infected with 10^4 PFU of FV3 in APBS. Plaque assays were performed for peritoneal leukocytes, kidney, spleen and liver at 3 and 9 days post-FV3 infection.

For tadpole survival studies, stage 50 tadpoles (12/treatment group, *N*=12) were infected as above and monitored over the course of 60 days. Stage 50 tadpoles were used to ensure that animals do not reach metamorphosis during the experimental period. Tadpoles were checked twice daily and dead animals were immediately frozen and stored at -20°C for DNA isolation.

Statistical Analysis. Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's post hoc test. Two-sample F-test was performed on the A6 cell Mx1 gene expression data. Probability level of $P<0.05$ was considered significant. Vassar Stat was used for statistical computation (http://faculty.vassar.edu/lowry//anova1u.html).

RESULTS

Gene expression analysis of *X. laevis* type I and type III IFNs. To investigate the biological roles of type III interferons in ectothermic vertebrates, we identified a *X. laevis* IFNλ gene homolog and compared its expression by qPCR with the previously identified *X. laevis* type I IFN; here referred to as IFN (Fig 1). *X. laevis* tadpoles (developmental stage 54) exhibited significantly greater IFNλ gene expression than that of IFN in all examined tissues, with the exception of kidney and intestine (Fig. 1A). IFNλ transcript levels were highest in the spleen, liver, thymus and lungs; more modest in the kidney and gills; and the lowest in the intestine. Similar expression patterns were observed in metamorphs (stage 64), with the exception of significantly elevated kidney and decreased thymic IFNλ gene expression (Fig. 1B, D). The intestinal gene expression levels of the metamorphic type I and type III IFNs were comparable (Fig. 1B), The adult frog type III IFN gene expression was also significantly higher than that of the type I IFN for all tissues examined, excluding intestine (Fig. 1C).

A comparison of type III IFN gene expression during *X. laevis* development revealed marked increases of IFNλ kidney and gill expression of this gene during metamorphosis over larval and adult stages (Fig. 1D). The considerable decrease in thymic IFNλ gene expression during metamorphosis, followed by its restoration in adult frogs is consistent with the death of most larval thymocytes during metamorphosis and the differentiation of adult thymocytes after the metamorphic completion [29]. In contrast, the decreased metamorphic lung IFNλ transcript levels persisted into frog adulthood (Fig. 1D).

Kidney IFNλ gene expression analysis in FV3-infected *X. laevis* tadpoles and adults. In our previous efforts to investigate the inefficiency in *X. laevis* tadpole antiviral immunity during the FV3 infections, we were perplexed to find that despite a meager and delayed type I IFN gene

expression response (as compared to adult frogs), tadpoles concomitantly exhibit significantly lower FV3 loads than *X. laevis* adults [14]. Given the overall greater IFNλ gene expression in tadpole tissues over the type I IFN, we hypothesized that IFNλ may play a more prominent role in tadpole antiviral immune responses. Accordingly, we examined the IFNλ transcript levels during FV3 infection in tadpole and adult frog kidneys (primary site of FV3 replication). Notably, although adult frogs displayed greater basal kidneys IFNλ transcript levels than tadpoles, IFNλ gene expression markedly increased (2 logs) as early as 24 hrs post-FV3 infection, whereas no significant expression increase was detected in infected adult kidneys (Fig. 2A). The IFNλ gene expression in tadpole kidneys remained elevated at 3 dpi and returned close to basal levels at 6 dpi (Fig. 2A). As previously observed, the FV3 genomic DNA copy number (as assessed by absolute qPCR) substantially increased in virally infected adult kidneys from 1 to 6 dpi, whereas the tadpole kidney FV3 loads were significantly more modest and did not increase 1 to 6 dpi (Fig. 2B).

Analysis of antiviral gene expression and anti-FV3 protection of A6 cultures stimulated with r*Xl*IFN or r*Xl*IFNλ. To determine whether the tadpole induction of IFNλ gene expression during FV3 infections could account for the relatively low FV3 loads, we generated a recombinant form of this cytokine (r*Xl*IFNλ) and compared its antiviral activity *in vitro* relative to the previously characterized recombinant *X. laevis* type I IFN (r*Xl*IFN) [14]. To assess the relative antiviral efficacies of r*Xl*IFNλ and r*Xl*IFN across a range of concentrations, we pretreated the kidney-derived A6 cell line cultures for 6 hrs with 0.5, 5, 50, 500 and 5000 ng/mL of either cytokine, infected them with FV3 and assessed the viral loads within these cultures by plaque assays (Fig. 3A). With the exception of the lowest tested doses, r*Xl*IFN proved to be more effective than r*Xl*IFNλ at preventing viral replication across all other tested concentrations (Fig.

3A). Notably, the tread-line for the dose dependent antiviral effects of r*Xl*IFN is substantially steeper (R^2=0.9692) than that for r*Xl*IFNλ (R^2=9457; Fig 3A). Based on our previous r*Xl*IFN studies [14] and in accordance to the dose-dependent antiviral effects of r*Xl*IFN and r*Xl*IFNλ presented here (Fig. 3A), we employed the intermediate 100 ng/mL doses of either cytokine for all subsequent *in vitro* studies. At this dose, qPCR analysis of FV3 DNA viral loads confirmed that although both recombinant cytokine markedly decreased viral loads in A6 cells, r*Xl*IFN was significantly more protective than r*Xl*IFNλ (Fig. 3B).

To account for the differences in anti-FV3 protection, we assessed antiviral gene expression in A6 cultures stimulated by either cytokine, during steady-state and following FV3 infection (Fig. 3C-G). A6 cells treated with r*Xl*IFN but not with r*Xl*IFNλ, exhibited increased type I IFN gene expression, and this was not significantly altered by FV3 infections (Fig. 3C). Remarkably, IFNλ but not type I IFN gene expression was induced by FV3 infection of A6 cells (Fig. 3C and D). Moreover, pretreatment of A6 cells with r*Xl*IFNλ resulted in further increases in IFNλ gene expression following FV3 infection (Fig. 3D). Conversely, although r*Xl*IFN induced its gene expression, FV3 infection did not significantly increase this r*Xl*IFN-mediated expression (Fig. 3C). It is of note that r*Xl*IFNλ pre-treatment did not induce type I IFN gene expression and vice versa (Fig. 3C and D).

The functional differences between the two IFNs were further evidenced by the distinct IFN-induced changes in Mx1, Mx2 and PKR gene expression responses (Fig. 3E-G). Pre-treatment of A6 cultures with r*Xl*IFN considerably increased the expression of the antiviral Mx1 and Mx2 genes without further significant expression changes observed following FV3 infection (Fig. 3E and F). By contrast, r*Xl*IFNλ pre-treatment resulted in significantly increased Mx1 but not Mx2 gene expression upon FV3 infection (Fig. 3E and F). Interestingly, FV3 infections dramatically

297 ablated the gene expression of protein kinase R (PKR) induced by both r*Xl*IFNλ r*Xl*IFN
298 pretreatments (Fig. 3G).

299 **Assessment of short-term r*Xl*IFNλ anti-FV3 protection in *X. laevis* tadpoles.** To extend
300 our *in vitro* findings, we administered r*Xl*IFN, r*Xl*IFNλ or the vector control intraperitoneally to
301 *X. laevis* tadpoles and examined antiviral gene expression in peritoneal leukocytes (PLs), kidney
302 (primary FV3 target) and spleen (central immune organ) 24 hrs later (Fig. 4). Interestingly,
303 r*Xl*IFNλ elicited robust Mx1 and Mx2 gene expression responses in PLs, whereas r*Xl*IFN
304 induced only a modest increase of Mx1 and no change in Mx2 mRNA levels (Fig. 4A and B,
305 respectively). Surprisingly, PKR gene expression was decreased in PLs from both r*Xl*IFN and
306 r*Xl*IFNλ-administered tadpoles (Fig. 4C).

307 In kidneys, r*Xl*IFN treatments induced marked increases in Mx1, Mx2 and PKR gene
308 expression, whereas r*Xl*IFNλ administration decreased Mx1 transcript levels and had no
309 significant effects of Mx2 and PKR expression (Fig. 4D-F). Finally, r*Xl*IFN treatment
310 significantly increased the splenic expression of Mx1, Mx2 and PKR, whereas r*Xl*IFNλ
311 decreased Mx1, but induced Mx2 (albeit significantly less so than r*Xl*IFN) and PKR expression
312 (Fig. 4G-I, respectively). These results further substantiate the functional differences between *X.*
313 *laevis* IFNλ and IFN in antiviral immune responses.

314 To further compare the antiviral effects of r*Xl*IFNλ and r*Xl*IFN, we next pretreated tadpoles
315 as above, infected them with FV3 and assessed FV3 viral loads in kidneys, PLs, spleens and
316 livers after 3 and 9 dpi by plaque assays (Fig. 5). As expected, the FV3 replication was markedly
317 higher in kidneys (over a log) than PLs, spleen or liver (Fig. 5), underlining the importance this
318 organ for both FV3 infections and thus tadpole anti-FV3 protection. Although pretreatment with
319 either recombinant cytokine resulted in similar protective effects in kidneys at 3 dpi (2 fold

decrease in virus load), prevention of viral replication by r*Xl*IFN was significantly more effective than r*Xl*IFNλ at 9 dpi. (Fig. 5A). For PLs, the protective effect of pre-treatment was only detected at 9 dpi and was not significantly different between the two recombinant cytokines (Fig. 5B). In the liver, FV3 loads were significantly diminished by r*Xl*IFN, but not r*Xl*IFNλ pretreatments, although viral load also decreased in vector treated control animals at 9 dpi compared to 3 dpi, suggesting the development of tadpole immune response more potent at limiting viral dissemination (Fig. 5C). Finally, in the spleen, only r*Xl*IFN pretreated animals showed significantly decreased FV3 loads at 9 dpi, whereas animals stimulated with r*Xl*IFNλ possessed significantly lowered spleen viral loads at 3 and 9 dpi, below levels detected in the r*Xl*IFN-cohorts (Fig. 5D).

It is to note that viral loads in kidney, liver spleen and peritoneal leukocytes of FV3-infected tadpoles pretreated with equal doses of the two recombinant cytokines were comparable to those following r*Xl*IFN treatments alone (data not shown), suggesting the absence of additive antiviral effects.

Assessment of long-term r*Xl*IFNλ anti-FV3 protection of *X. laevis* tadpoles. To further compare the antiviral effects of r*Xl*IFNλ and r*Xl*IFN, we next monitored tadpole survival following FV3 infection of control-, r*Xl*IFN- and r*Xl*IFNλ-stimulated animals (Fig. 6). Notably and consistent with the observed reduction of viral loads, both r*Xl*IFNλ and r*Xl*IFN treatments resulted in significant increases in tadpole survival, especially during the initial 25 days post-FV3 challenge. However, whereas the survival of r*Xl*IFN-stimulated tadpoles remained greater than that of control animals for the remained of the 60-day study, after 25 dpi the r*Xl*IFNλ-administered tadpole survival drastically decreased to levels comparable to those of vector control animals (Fig. 6A). Furthermore, while the r*Xl*IFN-treated animals had significantly

343 decreased post-mortem FV3 DNA loads, r*Xl*IFNλ-treated tadpoles possessed modestly but not
344 significantly diminished FV3 loads, as compared to vector control animals (Fig. 6B). These
345 results suggest that the anti-FV3 protection conferred by r*Xl*IFNλ is both less effective and
346 shorter-lasting than that of r*Xl*IFN.

347 **Analysis of IFNλ receptor gene expression in healthy and FV3-infected animals.** It is
348 well established that mammalian type III IFNs signal by ligating the interferon lambda receptor 1
349 (IFNLR1); subsequently complexed by the interleukin-10 receptor 2 (IL10R2), which propagates
350 the cellular signaling [6]. To more comprehensively define amphibian type III IFN antiviral
351 immunity, we examined the gene expression of the *X. laevis* IFNλ receptors in healthy and FV3
352 infected *X. laevis* tadpoles and adults (Fig. 7). The expression of both the IFNλ ligand binding
353 and signal propagating chains (IFNLR1 and IL10R, respectively) was significantly greater in
354 adult PLs, kidneys and especially spleens as compared to respective tadpole tissues (Fig. 7A and
355 B).

356 Intriguingly, IFNLR1 gene expression was significantly decreased in tadpole, but not adult
357 frog kidneys at 1 dpi, whereas at 3 and 6 dpi, both tadpoles and adults exhibited increased
358 IFNLR1 expression (Fig. 7C). This presumably reflects the previously observed timely leukocyte
359 infiltration of infected kidneys [25]. It is noteworthy that the increased kidney IFNLR1 gene
360 expression at 3 and 6 dpi was markedly lower in tadpoles than adult frogs (1 to 2 logs; Fig. 7C).
361 Interestingly, while tadpole spleen IFNLR1 gene expression significantly increased with
362 infection progression, the relatively robust adult splenic IFNLR1 levels significantly declined at
363 1 dpi and were restored by 3 dpi (Fig. 7D). Whether these splenic gene expression changes are
364 due to gene regulation and/or cell migration is currently unknown.

365 IL10R2 gene expression levels in kidneys and spleens of tadpole and adults were not

366 significantly altered under these experimental conditions and times examined (data not shown).

367 Since FV3 infections resulted in decreased tadpole kidney IFNLR1 gene expression (Fig.
368 7C), we also examined the IFNλ receptor gene expression in recombinant cytokine-stimulated,
369 FV3 infected A6 cultures (Fig. 8A). Notably, while FV3 infection significantly decreased the A6
370 cell expression of IFNLR1 and IL10R2, pretreatment of parallel cultures with either r*Xl*IFN or
371 r*Xl*IFNλ restored the expression of these two receptors in the face of FV3 challenge (Fig. 8A).

372 **Susceptibility of recombinant FV3 mutants deficient for putative virulence genes to**
373 **type I and III IFNs.** It stands to reason that the less-effective antiviral capacity of r*Xl*IFNλ, as
374 observed in our studies may be specific to FV3, a virus that has coevolved with the amphibian
375 immune system. This notion is supported by our findings that FV3 infections decreased IFNLR1
376 gene expression (Figs. 7C and 8A). To begin to address this issue, we took advantage of several
377 FV3 recombinant bearing site-specific deletions of putative virulence and/or immune evasion
378 genes. These knockout mutants virus included deletions for: a conserved ranavirus immediate-
379 early gene 18K (ORF 82R); a truncated viral homolog of the alpha subunit of eukaryotic
380 initiation factor 2 (eIF-2), vIF-2α (ORF 26R); and a viral protein with a Caspase Activation and
381 Recruitment Domain, vCARD (ORF 64R). Both FV3-Δ18K and FV3-ΔvIF-2α recombinants
382 were previously described and shown to contribute to FV3 virulence *in vivo* in tadpoles [27]. We
383 have recently generated an FV3-ΔvCARD recombinant that shows unaffected growth kinetics *in*
384 *vitro* in BHK cells (Andino *et al*., submitted). We hypothesized that one or several of these
385 deleted FV3 genes may target the antiviral effects elicited by IFNλ. Accordingly, A6 cultures
386 were pretreated with r*Xl*IFNλ, r*Xl*IFN, or vector control and then infected with WT or one of the
387 recombinant viruses (Fig. 8B). Notably, FV3-ΔvIF-2α and FV3-ΔvCARD but not FV3-Δ18K
388 showed a partial replication defect in A6 cells, and this defect was more pronounced by

pretreatment with either r*Xl*IFNλ or IFN (Fig. 8B). Interestingly, r*Xl*IFNλ was significantly more effective (P=0.008) against FV3-ΔvIF-2α and was as potent as r*Xl*IFN at inhibiting FV3-ΔvCARD replication (Fig. 8B). These results strongly suggest that vIF-2α and vCARD FV3 genes are critically involved resistance to IFNλ- and IFN-mediated antiviral responses, whereas 18K-mediated virulence is IFN-independent and here serves as an additional control.

DISCUSSION

This manuscript marks the first functional characterization of a type III IFN in an ectothermic vertebrate, the amphibian *Xenopus laevis*. Our findings are particularly relevant, considering the key position of amphibians in vertebrate phylogeny and evolution of antiviral interferon immunity. In this regard, a hallmark characteristic of fish and amphibian type I IFNs is the five exon/four intron genomic organization, not shared by the distinct intronless avian, mammalian and reptilian type I IFNs [10, 16, 17]. Moreover, in light of the complex evolutionary relationships of the teleost type I IFNs to higher vertebrate type I and/or type III IFNs [4, 15, 16, 30], the fact that amphibians possess both fish-like type I IFNs as well as *bona fide* type III IFNs [15] is particularly compelling. Provided that teleosts indeed do not possess type III IFNs, this implies that the divergence of type I and III IFNs took place prior to, or during the emergence of tetrapods [15] and brings to question the relative biological roles of the amphibian type I IFNs, as compared to those of fish. Here, we report that while an amphibian type III IFN appears to be less effective than a type I IFN in antiviral defense, this inefficiency may stem from a host-evasion strategy specific to FV3. Since a rapid and robust IFNλ gene expression is induced in *X. laevis* tadpoles in response to FV3, this cytokine may predominate antiviral defenses during early amphibian life. Moreover, our findings indicate that FV3 not only decreases kidney IFNLR1

gene expression early on during infection, but also counteracts the downstream antiviral cascades initiated by IFNλ. Thus, it is possible that, in comparison to the delayed and modest FV3-induced tadpole type I IFN expression [14], the prompt and robust IFNλ response in tadpoles but not adults may capsize the initial FV3 expansion, prior to FV3 host evasion, explaining the relatively modest tadpole FV3 loads. The current absence of *X. laevis*-specific anti-IFN and anti-IFNλ antibodies has prevented us from addressing whether the differences in gene expression correspond to differences in the respective IFN cytokine protein levels. It will be interesting to revisit this notion upon reagent availability.

It is interesting that that while both r*Xl*IFN and r*Xl*IFNλ elicited antiviral gene expression in the kidney-derived A6 cell line, the magnitudes of these expression changes were more prominent following r*Xl*IFN stimulation. Similarly, tadpole kidney and spleen expression of antiviral genes was more robust following r*Xl*IFN, as compared to r*Xl*IFNλ stimulation. By contrast, peritoneal leukocytes from r*Xl*IFNλ-administered animals exhibited substantially greater expression of Mx1 and Mx2. This is a bit paradoxical considering that our expression studies indicate that tadpole kidney and spleen tissues possessed greater IFNLR1 expression levels. Possibly, the kinetics of r*Xl*IFN- and r*Xl*IFNλ-elicited antiviral gene expression are distinct, whereby r*Xl*IFNλ may actually induce greater antiviral gene expression at distinct times. In support of this notion and in corroboration with the high splenic IFNLR1 expression, it is noteworthy that r*Xl*IFNλ-treated tadpoles actually exhibited significantly lower FV3 loads than the r*Xl*IFN-administered animals. Again, this brings to question the absolute efficacies of the *X. laevis* type I and type III IFNs since we observed FV3-induced downregulation of the tadpole kidney, but not splenic IFNLR1 expression; which correlate with the relatively less effective r*Xl*IFNλ protection of tadpole kidneys and more effective splenic protection.

435 Our previous investigations suggested that susceptibility of *X. laevis* tadpoles to FV3 was
436 marked by delayed and meager antiviral [14] and inflammatory [31] responses, as compared to
437 adults. The present evidence of rapid and greater IFNλ gene expression in response to FV3
438 infection warrants for a reevaluation of this hypothesis. It stands to reason, that tadpoles have an
439 intact and timely antiviral response in the form of IFNλ, which may be effective against less
440 proficient pathogens than ranaviruses. Indeed, r*Xl*IFNλ was as potent as r*X*IFN at inhibiting
441 FV3-ΔvCARD and even more potent at inhibiting the FV3-ΔvIF-2α recombinants. The
442 sensitivity of these two FV3 mutants to IFN response is also supported by their partially
443 defective replications in vector control-treated A6 cells compared to wild type or 18K knockout
444 FV3. In this regard, it is interesting that FV3 infection of A6 cells results is greater gene
445 expression of IFNλ than IFN.

446 These results are also interesting since the FV3 vIF-2α gene is truncated and lacks the
447 protein kinase R N-terminal binding and central helicase domains [27]. Non-the-less, ΔvIF2α
448 FV3 exhibits reduced replication and lower mortalities of infected *X. laevis* tadpoles [27], and
449 here is severely impaired in overcoming the antiviral effects of IFN and especially IFNλ.
450 Notably, several other ranaviruses including the Epizootic Haematopoietic Necrosis Virus
451 (EHNV, [32]); the Ambystoma tigrinum Virus (ATV, [33]); and the Rana catesteiana Virus Z
452 (RCV-Z, [34]) all encode full length vIF-2α genes. Moreover, both the ATV and the RCV-Z
453 vIF-2α gene products are thought to function as pseudo-substrates for the cellular protein kinase
454 R by inhibiting its phosphorylation of the cellular eIF-2α translation factor. While it remains
455 unclear whether the truncated vIF-2α may be expressed as chimeric product with an adjacent
456 ORF or whether it is capable of blocking PKR phosphorylation as a truncated protein, it is clear
457 that this truncated FV3 vIF-2α gene is critical for overcoming the IFN-induced antiviral state.

Substantially less is known regarding the ranavirus vCARD genes. The 10 kDa vCARD gene product contains a Caspase Activation and Recruitment Domain (CARD) motif that impairs interactions between other CARD-containing cellular proteins [35, 36]. Known cellular signaling moieties possessing such domains include pro-apoptotic proteins, pro-inflammatory molecules and most notably proteins participating in cellular interferon responses [37, 38]. It has been postulated that the ranavirus vCARD interacts with one or more of these signaling molecules to abrogate cellular antiviral responses and indeed our results indicate that the FV3 vCARD is crucial to overcoming cellular antiviral states induced by type I and type III IFNs.

It is interesting to consider that since tadpoles do not readily upregulate type I IFN expression, but undergo such drastic type III IFN gene responses to a viral infection, possibly ranaviruses coevolved to dampen the tadpole type III responses and the adult frog type I IFN immunity through virulence determinants such as vIF-2α and vCARD. Both the relative antiviral efficacy of r*Xl*IFN and the inefficiency of r*Xl*IFNλ against tadpole FV3 infections may reflect this. Indeed our observations that both cytokines are nearly equally effective at inhibiting FV3 kidney replication and tadpole survival earlier on in infection support this notion. Gaining further insights into the amphibian type I and type III IFN responses is imperative not only to defining the limitations within these immune mechanisms during ranaviral infections, but also to gaining a greater appreciation for the evolutionary origins of our own antiviral defenses.

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Figure Legends

FIG 1 *X. laevis* type I and type III IFNs tissue gene expression analysis. (A) Tadpoles (stage 54); (B) metamorphic froglets, (stage 64); and (C) adult frogs (2 years old). (D) Comparison of IFNλ tissue gene expression in pre-metamorphic, metamorphic and post-metamorphic *X. laevis*. Tissues from 3 individuals of each stage were examined (*N*=3). Above-head individual letters correspond to tissues exhibiting significantly different ($P < 0.05$) gene expression. The IFNλ tissue gene expression was significantly greater for all tissues with the exception of those denoted by •, ($P < 0.05$). The examined tissues include: K, kidney; S, spleen; M, muscle; In, intestine; L, liver, Th, thymus, Lu, lung, G, gill, BM, bone marrow. Gene expression was examined relative to the GAPDH endogenous control and all results are depicted as means ± SEM.

FIG 2 Quantitative analysis of (A) tadpole and adult *X. laevis* IFNλ gene expression and (B) kidney FV3 DNA loads at 0, 1, 3 and 6 days post infection. *X. laevis* tadpoles and adults were infected with $1x10^4$ and $5x10^6$ PFU of FV3, respectively. Tissues were isolated at indicated times and qPCR analysis performed to determine IFNλ gene expression relative to the GAPDH endogenous control and the FV3 loads in relation to an FV3 vDNA Pol II standard curve. Tissues from five individual animals (*N*=5) were assessed for each time point. Results are means ± SEM. Treatment groups resulting in significant differences are denoted by an (*), with the overhead line marking the respective treatments, $P<0.05$.

FIG 3 Assessment of the antiviral effects of r*Xl*IFN and r*Xl*IFNλ on the kidney-derived A6 cell line. (A) A6 cells were pretreated for 6 hrs with 0.5, 5, 50, 500 or 5000 ng/mL of either recombinant, infected at 0.5 MOI of FV3 for 16 hrs and assessed for viral loads by plaque assays. (B-G) A6 cultures were treated with the vector control or 100 ng/mL of either r*Xl*IFN or r*Xl*IFNλ for 6 hrs and infected with 0.5 MOI FV3 for an additional 16 hrs. (B) The FV3 DNA copy number was assessed by absolute qPCR against the FV3 vDNA Pol II (using a vDNA Pol II standard curve). Antiviral qPCR gene expression analysis included: (C) type I IFN; (D) type III IFN (IFNλ); (E) Mx1; (F) Mx2 and (G) PKR. Gene expression analysis (B-G) was performed relative to the GAPDH endogenous control. Three A6 cell cultures were subjected to each of the experimental conditions, *N*=3. Results are means ± SEM. The (*) denotes significant differences from the vector control and treatment groups resulting in significant differences are denoted by an (*), with the overhead line marking the respective treatments, $P<0.05$.

FIG 4 Assessment of the antiviral effects of r*Xl*IFN and r*Xl*IFNλ on *X. laevis* tadpole peritoneal leukocytes (A-C); kidneys (D-F); and spleens (G-I). Stage 54 tadpoles were ip injected with 1 µg of r*Xl*IFN, 1 µg of r*Xl*IFNλ or equal volumes of the vector control and antiviral gene expression was assessed 24 hrs later in peritoneal leukocytes (PLs), kidneys and spleens. The expression analyses included: (A) PLs Mx1; (B) PLs Mx2; (C) PLs PKR; (D) kidney Mx1; (E) kidney Mx2; (F) kidney PKR; (G) spleen Mx1; (H) spleen Mx2; and (I) spleen PKR. Gene expression was examined relative to the GAPDH endogenous control and all results are depicted as means ± SEM The (*) denotes significant differences from the vector control and treatment groups resulting in significant differences are denoted by an (*), with the overhead line marking the respective treatments, $P<0.05$.

FIG 5 Comparison of r*Xl*IFN and r*Xl*IFNλ anti-FV3 protection in tadpoles. Stage 54 tadpoles were ip injected with 1 µg of r*Xl*IFN, 1 µg of r*Xl*IFNλ or equal volumes of the vector control and infected 6 hrs later with 10^4 PFU of FV3. Viral loads were determined by plaque assays at 3 and 9 dpi for (A) kidneys; (B) peritoneal leukocytes; (C) livers; and (D) spleens. Four tadpoles (*N*=4) were employed for each treatment group. All viral loads are depicted as means ± SEM. The (*) denotes significant differences from the vector control and treatment groups resulting in significant differences are denoted by an (*), with the overhead line marking the respective treatments, $P<0.05$.

FIG 6 Survival of FV3-infected tadpoles pretreated with either r*Xl*IFNλ or r*Xl*IFN. Stage 50 tadpoles (12/treatment group; *N*=12) were pre-injected with 1 µg of r*Xl*IFN, 1 µg of r*Xl*IFNλ or equal volumes of the vector control and 6 hrs later infected with FV3 (10^4 PFU) or mock infected by APBS injections. (A) Animal survival was monitored over the course of 60 days post FV3 infection and (B) post-mortem viral loads were determined by absolute qPCR against the FV3 vDNA Pol II (using a vDNA Pol II standard curve). Results in (B) are mean FV3 DNA copies ± SEM. The (*) denotes significant differences from the vector control, $P<0.05$.

FIG 7 Gene expression analysis of the *X. laevis* IFNλ receptors, IFNLR1 and IL10R2. (A) IFNLR1 and (B) IL10R2 gene expression analysis in healthy (stage 54) tadpoles and adults (2 years old). Expression analysis of tadpole and adult (C) kidney and (D) spleen IFNLR1 at 0, 1, 3 and 6 days post FV3 challenge. Five animals (*N*=5) were used for each experimental group, all expression was performed relative to the GAPDH endogenous control and all results are presented as means ± SEM. The (*) denotes significant differences from the vector control and treatment groups resulting in significant differences are denoted by an (*), with the overhead line marking the respective treatments, $P<0.05$.

FIG 8 Assessment of A6 cell IFNλ receptor gene expression and r*Xl*IFN / r*Xl*IFNλ antiviral protection against recombinant FV3 A6 cells were pretreated with 100 ng/mL of r*Xl*IFN, 100 ng/mL of r*Xl*IFNλ, or equal volumes of the vector control for 6 hrs and infected with a 0.5 MOI of WT FV3 for 16 hrs before assessing (A) IFNLR1 and (B) IL10R2 gene expression by qPCR, using GAPDH as an endogenous control. (C) A6 cells were pretreated with 100 ng/mL of r*Xl*IFN, 100 ng/mL of r*Xl*IFNλ, or equal volumes of the vector control for 6 hrs and infected for 16 hrs with 0.5 MOI of either WT FV3, Δ18K FV3, ΔvCARD FV3, or ΔvIF-2α FV3. Cells were subsequently harvested, processed and assessed for respective viral burdens by plaque assays.

All experiments described above employed three A6 cultures per treatment group (N=3) and all of the results are presented as means ± SEM. The (*) denotes significant differences from the vector control. Treatment groups resulting in significant differences are denoted by an (*), with the overhead line marking the respective treatments. The statistically different protective effects conferred r*XI*IFN and r*XI*IFNλ against distinct recombinant FV3 are designated by 'a' and 'b'; representing relatively more and less significant protection, respectively. $P<0.05$.

Table 1. List of primer sequences

PRIMER	SEQUENCE (5'-3')
IFNλ (cloning)	F: ATGGAAATTCCTATCAGACTGGCCGCCATG
	R: TTCATTATTAGCCCAACACATTACATC
IFNλ (Insect expression)	F: GCTAAGCTTTCCACACAGAAGGCACTGCCACAT
	R: AGACTCGAGTTCATTATTAGCCCAACACATTAC
DNA Pol II	F: ACGAGCCCGACGAAGACTACA
	R: TGGTGGTCCTCAGCATCC T
GAPDH	F: GACATCAAGGCCGCCATTAAGACT
	R: AGATGGAGGAGTGAGTGTCACCAT
IFN	F: GCTGCTCCTGCTCAGTCTCA
	R: GAAAGCCTTCAGGATCTGTGTGT
IFNλ	F: TCCCTCCCAACAGCTCATG
	R: CCGACACACTGAGCGGAAA
IFNLR1	F: GGAGCCTGATCCCAATGAATTA
	R: TCTCAAAGCGCACACTAAGG
IL10R2	F: TCACCAGCATGGACTCTTTAC
	R: CTCACAAATGGCTTGGCTTAA T
MX1	F: AGCAGTGGTCAACAGGAGCC
	R: TGTTCCGCCGCTGTTCCTCT
MX2	F: GGAACGCCGCACTTGCAGAA
	R: CGATTAATCCTGGCACCTCC
PKR	F: GCTCACCGGCGGGATTA
	R: TTCAACTTTATTCATGCGTGCTAT C

F: Forward; R: Reverse