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***Xenopus-FV3* host-pathogen interactions and immune evasion**

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Abstract

We first review fundamental insights into anti-ranavirus immunity learned with the *Xenopus laevis*/ranavirus FV3 model that are generally applicable to ectothermic vertebrates. We then further investigate FV3 genes involved in immune evasion. Focusing on FV3 knockout (KO) mutants defective for a putative viral caspase activation and recruitment domain-containing (CARD)-like protein (64R-FV3), a β -hydroxysteroid dehydrogenase homolog (52L-FV3), and an immediate-early 18kDa protein (FV3-18K), we assessed the involvement of these viral genes in replication, dissemination and interaction with peritoneal macrophages in tadpole and adult frogs. Our results substantiate the role of 64R and 52L as critical immune evasion genes, promoting persistence and dissemination in the host by counteracting type III IFN in tadpoles and type I IFN in adult frogs. Comparably, the substantial accumulation of genome copy numbers and exacerbation of type I and III IFN gene expression responses but deficient release of infectious virus suggests that 18K is a viral regulatory gene.

Keywords

Amphibian; ranavirus; macrophages; virulence genes

Introduction

Over the last 50 years, infections caused by ranaviruses (*Iridoviridae*) in most part of the world have markedly increased in prevalence as well as in the range of species infected (Chinchar et al., 2009; Duffus et al., 2015; Kik et al., 2011; Kolby et al., 2014; Kolby et al., 2015; Price et al., 2014). Emerging infectious diseases caused by ranavirus are not only alarming for biodiversity and aquaculture, but also poses fundamental issues related to the evolution of host/pathogen interactions (Chen and Robert, 2012; Collins; Daszak et al., 1999; Gray et al., 2009; Robert and Gregory Chinchar, 2012). With regards to host immune response, it is startling that ranaviruses are capable of crossing species barriers among a

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wide range of ectothermic vertebrates including species from different classes of vertebrates ranging from amphibians to fish and reptile species. Such a promiscuous infectious ability suggests that these pathogens possess potent immune evasion mechanisms (Johnson et al., 2008; Mao et al., 1999; Robert and Jancovich, 2016). Furthermore, although some host species are highly susceptible to ranavirus, others are relatively resistant and can serve as asymptomatic carriers that disseminate infectious virus (Hoverman et al.; Robert et al., 2007; Teacher et al., 2009). This implies the involvement of host specific factors that determine the outcome of infection.

In mammals, the understanding of host immune responses to viruses has been by large derived from the mouse model (review in (Mahalingam et al., 2000; Panchanathan et al., 2008)). To a similar extent, the African clawed frog, *Xenopus laevis*, has provided and still serves as an instrumental model to gain insights into amphibian host-ranavirus pathogen interaction and immune evasion. In a relative short period of time, the experimental platform using *X. laevis* and the ranavirus Frog Virus 3 (FV3) has permitted to characterize critical mechanisms of host immune defenses to ranavirus. This body of work can serve as a foundation for anti-ranaviral immunity in amphibian and more generally cold blooded vertebrates (reviewed in (Chen and Robert, 2011; Chinchar and Waltzek, 2014). This model system takes advantage of the extensive characterization of the immune system of *Xenopus* and the availability immunological reagents such as antibodies developed for *X. laevis* (Robert and Ohta, 2009) as well as the large genetic and genomic resources (Xenbase), which includes the full annotated genome sequence of *X. laevis* (Session et al., 2016) and sister species *X. tropicalis* (Hellsten Uffe, 2009). We provide first here an updated review of what has been learned about immunity to ranavirus using the *Xenopus laevis*/ranavirus FV3 model system, before focusing on three putative ranavirus virulence genes.

1. Amphibian adaptive immune responses to ranavirus pathogens

As a preamble, studies in *Xenopus* have revealed a remarkable conservation at the functional level in host immune response against ranavirus, not only with DNA viruses in fish (Cuesta and Tafalla, 2009; Somamoto et al., 2014), but also with DNA viruses in mammals (Panchanathan et al., 2008). Thus, although species-specific variations and adaptations are expected, antiviral mechanisms are fundamentally conserved among cold blooded and warm-blooded vertebrates. In amphibians as in all jawed vertebrates, control and clearance of viral infection requires an efficient and timely collaboration and integration of both innate and adaptive arms of the immune system.

1.1. Adult frogs—Studies in *Xenopus* have demonstrated that upon a primary infection, adult frogs develop an active adaptive CD8⁺ T cell response. This response is characterized by an expansion of CD8⁺ T cells that peak at 6 days post-infection in the spleen and by corresponding increased infiltration of CD8⁺ T cell effectors in the kidney at the height of viral replication (Morales and Robert, 2007). These CD8⁺ T cells are required for subsequent viral clearance that typically occurs within 2 weeks following infection and adult host survival (Robert et al., 2005). Although, direct evidence of an anti-FV3 CD4⁺ T helper cell response is lacking, the role of CD4⁺ T cells in *Xenopus* antiviral response can be inferred by an expansion and infiltration of T cells recognized by the pan T cell marker CD5

that are CD8 negative, and by the production of effective IgY anti-FV3 antibodies able to inactivate FV3 *in vitro* (Chinchar and Waltzek, 2014; Maniero et al., 2006; Robert et al., 2005). The production of IgY antibodies requires T cell help (likely CD4⁺) for the isotype switch from IgM (Blomberg et al., 1980). Interestingly, as in the case of anti-pox immune response in mammals (Panchanathan et al., 2006, 2008), detectable potent IgY antibody response to FV3 in *Xenopus* only occurs during a secondary FV3 infection, despite the induced expression of the B cell specific activation-induced cytidine deaminase (AID) mediating isotype switch as early as 3 days following primary FV3 infection (Marr et al., 2007). Notably, B cell memory established during the primary infection, could be detected for at least 6 months after this primary infection (Maniero et al., 2006). This suggests that adult frogs surviving a primary ranavirus infection can remain resistant to a subsequent infection for long time. Indication of immunological memory leading to increased survival and/or improved viral clearance has been reported in turtles (Hausmann et al., 2015). Evidence documenting an active adaptive immune response against ranavirus infection is also emerging from recent transcriptome studies in the non-model amphibian *Rana temporaria* species (Price et al., 2015). Of further interest in this case, is the lack of differential expression for many immunologically-related genes upon ranavirus infection, which is perhaps due to the use of metamorphic animals. In *Xenopus*, it is well established that it take 4 to 6 weeks after the metamorphic completion for full recovery of immune functions (Robert and Ohta, 2009).

1.2. Tadpoles—*Xenopus* as other anuran species is characterized by a larval stage (tadpole) during which the immune system is generally considered to be more immature than adults, with a weaker antibody response, a poor isotype switch from IgM to IgY and weaker adaptive T cell response (Robert and Ohta, 2009). Consistent with this, tadpoles are usually more susceptible to ranavirus infection (Bayley et al., 2013; Landsberg et al., 2013; Reeve et al., 2013). In *X. laevis*, tadpoles are typically unable to control FV3 infection and most of them succumb. However, death from FV3 infection occurs gradually over 1 to 2 months, which is quite a long time (Gantress et al., 2003; Robert et al., 2005). In addition, we have noted that death is not always correlated with a high viral load (Grayfer et al., 2015). In fact, compared to adult frogs, viral loads in tadpole tissues including the kidney are significantly lower than adults even at early stages of infection, which suggest a distinct host-pathogen interaction between FV3 and the immune system of either tadpole or adult frogs. As such, it is important to remember that, despite some weaknesses, tadpoles are not just immunologically ignorant or deficient but rather have a distinct set of immune responses adapted for their life stage (Robert and Ohta, 2009). This, in turn, would imply that differences in selective pressure have lead FV3 and other ranaviruses to adapt their infection strategies for adult and larval stages. Indeed, adult and larval frogs have different ecological niches and have, therefore, evolved different approaches to fighting viral pathogens.

1.3. Importance of innate-like T cells in amphibian host defenses against ranavirus—Besides a minor fraction of conventional CD8⁺ T cells and MHC class II-restricted T cells (likely CD4⁺ T helper), the tadpole adaptive immune system is dominated by six subsets of innate-like (i)T cells representing about 80% of CD8 negative and CD8^{low} lymphocytes (Edholm et al., 2013; Robert and Edholm, 2014). These iT cells express a very

limited or invariant T cell receptor repertoire and require non-polymorphic MHC-like molecules rather than classical MHC class I molecules for their development and function (Edholm et al., 2013). Of particular relevance for ranavirus immunity, we found that one iT cell subset expressing the invariant rearrangement V α 6-J α 1.43 restricted by the MHC class I-like molecule XNC10 is critical for anti-FV3 response in tadpoles (Edholm et al., 2013). The loss-of-function of the *mhc1b10.L* gene encoding the XNC10 molecule established by combining RNA interference with transgenesis, abrogates the development of V α 6 iT cells. This *XNC10* loss-of-function markedly increases tadpole susceptibility to FV3 infection, resulting in increased viral replication and high lethality at early stage of infection (Edholm et al., 2013). V α 6 iT cells are also important in adult host response as evidenced by the delay in antiviral response, increased viral load and kidney damage (Edholm et al., 2015). However, the mature adult immune system is still sufficient to ultimately control the viral infection and clear FV3.

2. Amphibian innate immune responses to ranavirus

Although the innate arm of the immune system is often considered as ancillary, only assisting the adaptive arm of the immune system, its central role in host antiviral resistance has become appreciated. In adult *Xenopus*, FV3 infections rapidly (as early as 1 day post-infection) induce potent type I interferon (IFN) response and interferon response factors (Mx1, Mx2) as well as pro-inflammatory or inflammatory-associated (TNF α , IFN γ , IL-1 β) responses in parallel with the recruitment of activated macrophages to the site of infection (De Jesus Andino et al., 2012; Grayfer et al., 2014a, 2015). In contrast, the induction of pro-inflammatory immune gene expression (TNF α , IFN γ , IL-1 β) by FV3 in tadpoles is delayed and of lower magnitude compared to adult frogs (De Jesus Andino et al., 2012). In addition, there is a poor recruitment of granulocyte-colony stimulating factor (G-CSF) receptor (G-CSFR) expressing granulocytes into tadpole kidneys, consistent with the weak inflammatory response to FV3 (Koubourli et al., 2017). However, it would be too simplistic to conclude that tadpole antiviral immunity is inefficient.

2.1. Interferon response—Type I IFN has clearly potent antiviral activity as demonstrated by its ability to protect for FV3 infection in the *Xenopus* A6 kidney cell line pretreated with a *X. laevis* recombinant type IFN *in vitro* and by partially protecting pretreated tadpoles subsequently infected with FV3 (Grayfer et al., 2014a). The interferon system in *Xenopus* as in mammals and birds also includes type III or IFN- λ , which is more prominently involved in tadpoles than adult frogs during FV3 infection (Grayfer et al., 2015). Pre-injection of recombinant IFN- λ , can also confer some protection against FV3 infection in tadpoles, but not as efficiently as type I recombinant IFN (Grayfer et al., 2015). This is possibly because FV3 infection readily impairs IFN- λ , receptor gene expression in tadpoles and in *X. laevis* A6 kidney cell lines. Interestingly, recent evidence using water exposure to FV3 as a natural route of infection of skin mucosa has revealed a distinctive reliance on IFN between a *X. laevis* adult predominant type I-based and a tadpole mainly type III-based antiviral IFN systems (Wendel et al., 2017). In addition, the rapid decrease of viral loads in tadpole skin mucosa over 72 hr post-water infection indicate an active and efficient antiviral response (Wendel et al., 2017). Therefore, as in the case of adaptive immunity, it is likely that in response to developmental and evolutionary pressures, the

regulation and activation of the innate components of antiviral immunity in tadpoles is distinct from that of adult frogs.

2.2. Complex role of macrophages—A key immune effector cell type in host response to FV3 in both tadpoles and adult *X. laevis* are monocytic phagocytes of the myeloid lineage or macrophages. Compared to mammals where many types of tissue resident and circulating macrophages have been identified, the understanding of distinct macrophage subsets in cold blooded vertebrates, including *Xenopus*, is limited. However, monopoiesis is fundamentally conserved between amphibians and mammals (reviewed in (Grayfer and Robert, 2016; Huber and Zon, 1998; Robert and Ohta, 2009)). Macrophages reside in most organs and tissues throughout the amphibian body, where they provide a core foundation of the first line of host immune defenses against infectious agents such as ranavirus. Indeed, macrophages play a key role in orchestrating antiviral immunity against FV3 (Grayfer and Robert, 2016). Notably, the macrophage-colony stimulating factor (CSF) receptor 1 (CSF-R1) that drives the differentiation and function of macrophages was shown to interact in *Xenopus* as in mammals, with two distinct, evolutionarily unrelated ligands, CSF-1 and Interleukin 34 (IL-34; (Grayfer et al., 2014b)). Using recombinant *X. laevis* proteins, we showed that, both in adults and tadpoles, macrophages stimulated *in vitro* or *in vivo* by IL-34 exhibited stronger antiviral activity characterized by the production of antimicrobial factors (iNOS) and by preventing viral replication when infected with FV3 (Grayfer and Robert, 2014, 2015). In contrast, CSF-1 derived macrophages were more phagocytic and, as a possible consequence, more susceptible to FV3 infection.

This functional disparity is of particular relevance since macrophages are not only crucial antiviral effector cells but appear to be specially targeted by ranavirus pathogens in *Xenopus* and other species including mammals (Gendraut et al., 1981; Grayfer and Robert, 2016; Gut et al., 1981). Considering the indiscriminate infection mode and apparent absence of specific cellular receptor(s) requirement, we have proposed that macrophages with their ability to acquire exogenous particles and antigens through various pathways such as micropinocytosis and phagocytosis represent an advantageous cellular target for viral dissemination and persistence into the host. Consistent with this postulate, dissemination of FV3 into the *X. laevis* tadpole brain is associated with and can be promoted by macrophages infiltration into brain tissue (De Jesus Andino et al., 2016). Furthermore, accumulating evidence indicates that macrophages are critical for viral persistence in asymptomatic adult *X. laevis* (Robert et al., 2007). FV3 infecting peritoneal macrophages *in vivo* is found to become rapidly transcriptionally silent or quiescent (Morales et al., 2010). Similarly, little to no replication of FV3 occurs following *in vitro* infection of peritoneal macrophages despite FV3 efficient penetration into the cells as indicated by the viral load recovered at early stage of infection (Robert et al., 2014). This non-permissiveness of viral replication is enhanced in IL-34-derived (Grayfer and Robert, 2014, 2015). The possible role of macrophages as reservoirs for viral persistence in resistant hosts is of relevance given that ranavirus is increasingly detected in asymptomatic amphibian populations in the wild (Brenes et al., 2014; Forzan and Wood, 2013) as well as in captive turtle species (Hausmann et al., 2015). The threat of resistant animal carriers in propagating ranavirus infection is underscored by the fact that *X. laevis* adults can harbor quiescent virus prone to reactivation. This was

demonstrated by provoking inflammation with heat-killed bacteria in adult *X. laevis* one month past the time of viral clearance of a primary infection, as a way to reactivate FV3 (Robert et al., 2014). This treatment of apparently healthy animals not only induces the reappearance of active FV3 infection in peritoneal macrophages, but also results in significant increase in mortality from systemic viral infection. The increased susceptibility of previously infected, but apparently healthy, adults frogs to reactivated FV3 infection suggests that conditions altering the immune status such as stress or pollution can contribute to the dissemination of emerging ranavirus infections. In this regard, we have reported that exposure during early development of tadpoles to water contaminants including the herbicide atrazine and the insecticide carbaryl, even at very low subtoxic doses, induces long lasting defect of antiviral immunity that persists into adults (De Jesus Andino et al., 2017; Sifkarovski et al., 2014). For example, exposure of tadpoles for only 3 weeks to low (0.1 and 1.0 ppb) subtoxic concentrations of carbaryl induced long lasting defects of expression response of pro-inflammatory (IL-1 β) and antiviral type I interferon (IFN) genes persisting after metamorphosis (De Jesus Andino et al., 2017). This was accompanied by a significant increase in viral loads in infected tissues of young adult frogs several months after being exposed to the contaminant.

3. Ranavirus genes involved in immune evasion

The other side of the host-pathogen equation is represented by the ranavirus pathogens themselves, and more specifically by their putative virulence and immune evasion genes. As large double stranded DNA viruses, ranavirus genomes encode as many as 100 genes, the majority of which are of unknown functions and do not share any sequence similarity outside iridoviruses (Chinchar et al., 2009). We and others have developed a convenient and reliable method for generating ranavirus recombinant by site-specific integration of a fluorescent gene (GFP) reporter fused to a drug resistance gene (puromycin or neomycin) under the control of the immediate early 18K FV3 promoter (Chen et al., 2011; Jancovich and Jacobs, 2011). To date, the putative immune evasion function of the viral homolog of the cellular translation factor eIF-2 α (vIF-2 α) that can antagonize protein kinase R (PKR) is one of the best documented examples (Jancovich and Jacobs, 2011; Rothenburg et al., 2011; Rothenburg et al., 2008).

Among the potential other FV3 immune evasion gene candidates for which homology to eukaryotic gene could be inferred, two have retained our attention: 64R encoding a Caspase-like Activation and Recruitment Domain decoy (vCARD)-like molecule; and 52L encoding a β -hydroxysteroid dehydrogenase (β HSD)-like molecule. Using the technique described above we generated FV3 knock-out (KO) recombinants for 64R and 52L (Andino Fde et al., 2015). Because of its robust immediate early expression pattern and dispensable role for productive infection in cell culture (Cheng et al., 2014; Sample et al., 2007), the FV3 ICP18 or 18K (82R) gene was also selected as a virulence gene candidate and knocked out (Chen et al., 2011). Evidence from initial characterization of two FV3 KO viruses (64R-, and 52L-FV3) is consistent with their involvement in immune evasion, whereas results obtained with 18-FV3 suggest a more complex role of the 18K gene. While deficiency of each of these viral genes results in different degrees of attenuated viral replication in *X. laevis* hosts, closer examination *in vitro* indicates distinct, albeit not fully defined, functions. Infection

studies of A6 kidney cells suggest that the CARD-like molecule encoded by 64R interferes with IFN-induced apoptosis, whereas 18K may indirectly alter IFN responses by regulating other viral genes (Andino Fde et al., 2015). The role of the β HSD-like molecule encoded by 52L has remained elusive to date. Figure 1 summarizes what has been learned to date about *FV3-Xenopus* host pathogen interactions contrasting the adult and tadpole stages.

To further elucidate the role of these 3 putative immune evasion genes, we have assessed in detail replication, dissemination and persistence of each FV3 KO in tadpole and adult *X. laevis*.

Results

Effects of vCARD (ORF64R) and β -HSD (ORF52L) gene deletions on FV3 infectivity in *X. laevis* tadpoles

We previously reported that deletion of either 64R or 52L genes reduced FV3 replication in tadpole kidneys, which is the main site of viral replication in *X. laevis* (Gantress et al., 2003). Since larval peritoneal macrophages are particularly susceptible to FV3 infection (De Jesus Andino et al., 2012) and that FV3 is able to disseminate in tadpole brain (De Jesus Andino et al., 2016), we thought to first examine the ability of knock-out (KO) recombinant FV3 64R- or 52L-FV3 compared to FV3-WT to actively infect these sites and induce host antiviral responses. Accordingly, we determined the viral loads and expression of several key host antiviral genes in peritoneal leukocytes, brain and kidneys of tadpoles at different times postinfection. Defects were more dramatic for 64R-FV3, which was barely detected in PLs and brain at all time points tested, suggesting a weak ability to infect and persist in infected peritoneal cells as well as a poor dissemination (Fig. 2). In kidney, the increase in viral load was markedly delayed and became detectable only for a fraction (3 individuals from each experiment) of animals at 6 dpi, whereas it stayed close to base line levels the remaining tadpoles. In all animals tested, the 64R-FV3 genome copy number reached similar level to WT-FV3 at 12 dpi. Unlike 64R-FV3, 52L-FV3 disseminated more efficiently in the brain, but was significantly impaired in persisting in PLs and in replicating in kidneys compared to FV3-WT. It is noteworthy that viral loads in each group exhibited substantial individual variability especially at early time points (2 and 6 dpi). This has been observed before (De Jesus Andino et al., 2012) and may reflect both experimental errors (small variations in the infectious doses injected in each animals by the investigator) and genetic variations of the hosts since outbred animals were used. Importantly, however, comparable variability in each group was observed in the two experiments that were combined to achieve statistical significance.

The distinctive defects associated with 64R and 52L deletions were further revealed by the changes in expression of critical innate immune genes in tadpole kidneys (Fig. 3). Consistent with the low viral loads, gene expression responses for TNF α was ablated in kidneys of tadpoles infected with 64R-FV3, and significantly reduced for type I IFN at 2 and 6 dpi. In addition, IFN- γ (type II) gene expression was significantly decreased at 6 dpi in 64R-FV3 compared to FV3-WT. However, the 64R deletion did not significantly affected IFN- λ , gene response in kidney, which is a critical antiviral response in tadpoles. For 52L-FV3, there was only a significant diminished type I IFN gene expression at 2 dpi compared to FV3-WT.

In tadpole brains, while 64R or 52L deficiency did not result in statistically significant alteration the inflammatory gene TNF α expression response compared to WT-FV3, type I IFN gene expression was exacerbated at 6 dpi and remained high at 12 dpi in tadpole infected with 64R-FV3 (Fig. 4). Enhanced type I IFN gene expression was also triggered by 52L-FV3 at late stage of infection (12 dpi). In contrast, the two KO FV3 recombinants induced significantly less increase of IFN- λ , gene expression than WT-FV3 at 2 and 6 dpi, whereas only 52L-FV3 infection had a significant defect in the IFN- γ gene response at 6 dpi (Fig. 4). Thus, 64R and 52L deletions result in different alteration of IFN gene response in kidneys and brain. No significant changes in the gene expression patterns was observed in PLs at 2 and 6 dpi for the different KO FV3, although there was a high individual variability (Suppl. Fig 1).

Effects of 18K gene deletion on FV3 infectivity and IFN response in *X. laevis* tadpoles

To date, the potential function of the immediate-early 18K gene remains unclear. However, the absence of sequence similarity with any eukaryotic or prokaryotic genes compared to the high degree of conservation of this gene within the ranavirus genus suggests a biologically important species-specific role of 18K molecules. Based on our previous studies with a 18K-FV3 KO mutant, we postulate that 18K play a role in immune evasion by interfering with the IFN response (Andino Fde et al., 2015). We further examined the ability of 18K-FV3 to infect tadpole PLs, replicate in kidney and disseminate into the brain. In contrast to 64R-FV3 and 52L-FV3, replication of 18K-FV3 in kidneys, as determined by genome copy number using absolute qPCR was minimally affected with a slight but no statistically significant decrease at 2 dpi compared to WT-FV3 in this group of animals (Fig. 2). Interestingly, higher 18K-FV3 than WT-FV3 genome copy number was detected at late stage (12 dpi) of infection. Infection in PLs and brain was similar between 18K-FV3 and WT-FV3.

Reminiscent to what was observed *in vitro* using the A6 kidney cell line (Andino Fde et al., 2015), 18K-FV3 had the tendency to exacerbate tadpole IFN responses. In PLs, although high individual variation prevented to reach statistical significance, there was a trend toward higher expression of type I and type II IFN genes at 2 dpi in 18K-compared to WT-FV3 infected tadpoles (Suppl. Fig. 2). In kidneys, however, both type I and type III INF gene expression response was significantly increased at early stage of infection (2 dpi) with 18K-compared to WT-FV3 (Fig. 5A). Similarly, significantly higher type I IFN gene expression was triggered by 18K-FV3 than WT-FV3 the brain at 6 but not 2 dpi (Fig. 5B). In addition and in contrast to what was observed in PLs and kidneys, gene expression of type III IFN in the tadpole brain was significantly reduced in 18K-FV3 compared to WT-FV3 at 2 dpi.

Comparably, TNF α gene expression induced by 18K-FV3 was significantly reduced compared to WT-FV3 at 6 dpi in kidneys while no statistical difference was observed in the TNF α gene expression in the brain (Fig. 5 and Suppl. Fig. 2).

Infectivity KO-FV3 in *X. laevis* adults

To date the consequence of specifically deleting 64R, 52L and 18K genes on FV3 infectivity has been mainly characterized in *X. laevis* tadpoles, which are more susceptible than adult frogs and, as detailed in the introduction, rely on distinct antiviral immunity. Therefore, we were interested to determine in more detail the infection and replication efficiency of 64R-, 52- and 18K-FV3 in adult frogs.

For 64R-, 52-FV3, while as previously reported the replication of these 2 KO mutant FV3 was impaired in adult kidneys (Andino Fde et al., 2015), the capacity to infect PLs at early (1 dpi) and later (6 dpi) stage of infection was also significantly reduced (Fig. 6). For 18K-FV3, the genome copy number was also significantly reduced in PLs at 1 and 6 dpi. Although 18K-FV3 viral load was lower at 6 dpi in kidney, it did not reach statistical significance in this experiment (Fig. 6). This may indicate some variability since in an independent smaller experiment, 18K-FV3 infection result in significantly lower genome copy numbers compared to WT-FV3 (Suppl. Fig 3). The drastic replicative impairment of 64R-FV3 and more moderate defect of 52L-FV3 are reflected in the number of infectious particles recovered from kidneys of infected tadpoles at 6 dpi (Table 1). Intriguingly, however, despite a relatively high genome copy number in experiment of Fig. 6, we barely detected any infectious 18K-FV3 by plaques assay at 6 dpi in the kidney of 6 animals in two independent experiments (Table 1). Moreover, consistent with a possible defect in the final generation of infectious viral particles, expression of the late viral gene MCP, an essential gene, was significantly reduced in 18K-FV3 infected kidney at 6 dpi (Fig. 7).

The induction of antiviral gene expression responses was not significantly altered at 6 dpi in PLs and kidneys of adult infected with either 64R- or 52-FV3 when compared to WT-FV3. However, the examination of host antiviral gene expression revealed that infection with 18K-FV3 in adults as in tadpoles resulted in an exacerbated expression of type I as well as type III INF genes at 6 dpi both in kidneys and PLs (Fig. 8).

Discussion

Identifying and deciphering the function of viral genes that enable ranavirus pathogens to overcome host immune responses is in its infancy. The establishment of reliable methodologies to generate ranavirus recombinant deficient for specific ORFs constitutes a first critical step that not only permits the identification of non-essential genes candidates contributing to virulence or immune evasion but also provide stable deficient virus recombinant invaluable for uncovering mechanisms by which ranavirus interfere with host defenses. Although, investigation *in vitro* with host cell lines cultures can provide useful information, the complexity of the immune system ultimately requires *in vivo* studies using whole animals such as the amphibian *Xenopus*. Building upon our previous characterization of KO FV3 recombinants, here we have further investigated the infection patterns and host immune responses to 3 KO FV3 recombinants 64R, 52- and 18K -FV3, both in tadpoles and adult frogs. Our data substantiate our initial, mainly *in vitro*, characterization, suggesting that these viral genes play distinct non-overlapping roles in promoting successful infection of *X. laevis* hosts.

64R vCARD-like encoding gene

Typically in eukaryotic cells CARD motifs are involved in interactions among various CARD-containing cellular proteins (Kawai and Akira, 2009, 2010). Cellular signaling molecules containing CARD domains of particular interest in antiviral responses include pro-apoptotic proteins, pro-inflammatory molecules and proteins participating in the cellular interferon responses (Besch et al., 2009; Meylan et al., 2005). Based on sequence similarity, the ranavirus vCARD-like protein could interact with one or several of these signaling molecules to circumvent the cellular antiviral responses. Our study using the *X. laevis* A6 kidney cells lines showed that in absence of the 64R gene encoding vCARD, FV3 was more susceptible to both type I and III IFN response and induced more apoptosis, suggesting that vCARD contributes in subverting the antiviral IFN response within infected cells (Andino Fde et al., 2015; Grayfer et al., 2015). But because as much IFN is induced by the vCARD KO mutant as WT virus, it was concluded that vCARD may not directly interfere with type I IFN synthesis (Andino Fde et al., 2015). In the present study, considering that IFN- λ gene expression in tadpoles at the site of infection in PLs and at the primary site of infection in kidneys is mostly unaltered, it is tempting to conclude that vCARD is critical for FV3 to overcome the tadpole IFN- λ antiviral system, which is prominent over type I IFN in tadpoles. The weaker type I IFN gene expression response induced by 64R-FV3 is likely related to the low viral loads. 64R-FV3 replication and persistence is also impaired in adult. However, it is interesting to note the higher viral load in adult PLs compared to tadpoles. The significant decrease in genome copy numbers in adult kidneys at 6dpi compared to adult PLs suggests a faster clearance of 64R-FV3. The higher type IFN gene response resulting from 64R-FV3 in the tadpole brain is more puzzling given the barely detectable viral load and IFN- λ gene expression. Further study will be needed to determine whether this could be due to some secondary effect deregulated viral gene expression and/or tissue specific effect.

52L v β HSD-like encoding gene

The ranavirus homolog of β -hydroxysteroid dehydrogenase (v β HSD) has been identified as another possible immune evasion protein. The eukaryotic gene β -hydroxysteroid dehydrogenase is required for the synthesis of progesterone, mineralocorticoids, and glucocorticoids (GCs; (Rhen and Cidlowski, 2005)). β HSD homologs are present within poxviruses and have been shown to play a role in dampening host immune responses (Moore and Smith, 1992; Sroller et al., 1998; Sun et al., 2006). Whether the putative ranavirus homolog of β HSD functions in the same way remains to be fully determined. Constitutive expression of the *Rana grylio virus* (RGV) β -HSD homolog slightly suppressed cytopathic effect and prolonged the viability of infected cells (Sun et al., 2006). Although, similar to vCARD, deletion of v β HSD encoded by 52L gene reduces the ability of FV3 to infect *X. laevis* A6 kidney cells *in vitro* and tadpole *in vivo*, the loss of v β HSD did not markedly increased apoptosis of infect A6 cells (Andino Fde et al., 2015). The present study provides further evidence of the distinct roles between vCARD and v β HSD in promoting FV3 infection. FV3 lacking v β HSD is impaired in viral replication, dissemination and persistence, albeit not as dramatically as vCARD deficient FV3.

The last putative virulence or immune evasion FV3 gene examined in this study is the enigmatic 18K or ICP18 gene. In FV3 as in other virus, this is an immediate early gene

(Cheng et al., 2014). While the function of 18K remains unknown, its abundance and temporal class suggests that it is likely an important viral regulatory protein. Phylogenetic analysis of the amino acid sequence of the FV3 18K open reading frame indicates that it is conserved among members of the genus *Ranavirus*, but is not found among other genera (*Lymphocystivirus*, *Megalocytivirus*, or *Iridovirus*) within the family. Collectively, these data indicate that the 18K gene encodes a protein unique to the genus *Ranavirus*. The 18 kDa immediate-early protein is thought to be non-essential for replication in BHK and FHM cells (Sample et al., 2007). However, *in vitro* study with the *X. laevis* A6 kidney cell line has shown that 18K-FV3 is as resistant to type I and type III IFN inhibition as WT-FV3, but also markedly induces type IFN gene expression and apoptosis (Andino Fde et al., 2015). In the present *in vivo* study, we also found that infection of both tadpole and adults *X. laevis* with 18K-FV3 exacerbate the expression of the type I IFN gene. Notably, replication of 18K-FV3 is not as impaired as the two other KO FV3 as indicated by the substantial genome copy number found in different tissues of tadpoles and adults frogs. However, in adult we barely detected the production of infectious viral particles in kidneys by plaque assay, suggesting that the final step of viral assembly was not effective. Defect in release of infectious virus is also supported by the significant down expression of the FV3 MCP gene that is essential for the synthesis of the formation of the viral capsid. These data are consistent with the idea that 18K defective FV3 exhibit a deregulated and overabundant production of viral genomes that may lead to a greater intracellular PKR detection/signaling and thus trigger increases in cellular apoptosis as well as an increase of IFN-I response. As such, 18K is a viral regulatory gene rather than a typical immune evasion gene.

Materials and Methods

Animals

Outbred (OB) pre-metamorphic *X. laevis* tadpoles (stage 54–56 / 3 weeks-old) and two-year adult frogs were obtained from our *X. laevis* research resource for immunology at the University of Rochester (<https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis.aspx>). All animals were handled under strict laboratory and University Committee on Animal Resources regulations (100577/2003–151), and discomfort was minimized at all times.

Animal Infections

Three weeks-old tadpoles (developmental stage 54–56; (Nieuwkoop and Faber, 1994)) were infected by intraperitoneal (i.p.) injection with 1×10^4 PFU of FV3 in 10 μ L volume of amphibian phosphate-buffered saline (APBS) using a glass Pasteur pipette with the extremity elongated by flame and attached to rubber tubing (Gantress et al., 2003). Adult frogs were infected by i.p. injection with 1×10^6 PFU of FV3 in 100 μ L volume APBS using a 1 ml sterile syringe with a 22 gauge, 1½ inch needle. Mock-infected controls (0 days post-infection, d.p.i.) were ip injected with the same amount of amphibian phosphate-buffered saline (APBS). Animals were euthanized by immersion in 1% tricaine methane sulfonate (TMS-222) buffered with bicarbonate. At the indicated times, peritoneal leukocytes were collected by peritoneal lavage alternatively, animals were euthanized by

immersion in 1% tricane methane sulfonate (MS-222), and tissues were removed and processed for RNA and DNA isolation.

Cell lines and FV3 Stocks

High titer of WT-FV3 (Granoff et al., 1965); ATCCVR-569), 64R-FV3, 52-FV3 (Andino Fde et al., 2015) stocks were produced using Baby hamster kidney-21 cells (BHK-21; ATCC no. CCL-10) that were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/mL) and streptomycin (100 µg/mL) with 5% CO₂ at 37°C. FV3 was grown by a single passage on BHK-21 cells, purified by ultracentrifugation on a 30% sucrose gradient. Virus was quantified by plaque assay on BHK-21 monolayers in 6-well plates under an overlay of 1% methylcellulose (Morales et al., 2010). Infected cells were cultured 7 days at 30°C in 5% CO₂. Overlay media was aspirated and the cells stained for 10 min with 1% crystal violet in 20% ethanol.

Genome copy number and gene expression analysis

Genomic DNA and total RNA were isolated from tissues or cells using Trizol reagent (Invitrogen) following the manufacturer's protocol. 1 µg total RNA was transcribed into cDNA with iScript reverse transcriptase using oligo-dT primers (Bio-Rad). Quantitative PCR parameters were as follows: 2 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantitative PCR (qPCR) gene expression analysis (type I IFN, type II IFN, type III IFN and TNF-α) was performed using the CT method. Expression of the different genes was examined relative to the endogenous GAPDH control and normalized against the expression of each gene compared to APBS injected control groups. Absolute qPCR was performed to measure FV3 viral loads in isolated genomic DNA, using a serially diluted standard curve, as previously described (Grayfer et al., 2014a). All primers were validated prior to use. All primer sequences are listed in Table 1.

Statistical analysis

All quantitative data were analyzed using by a one-way test of variance (ANOVA) followed by pos-hoc analysis using the Vassar Stat software (<http://faculty.vassar.edu/lowry//anovalu.html>). A *p* value < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The *X. laevis-FV3* model permits basic understanding of immunity to ranavirus
- 64R and 52L counteract type III IFN in tadpoles and type I IFN in adult frogs
- 64R and 52L are critical immune evasion genes in *Xenopus* adults and tadpoles
- 18K deletion exacerbates IFN responses and impairs release of infectious FV3
- 18K is a viral regulatory gene rather than immune evasion gene

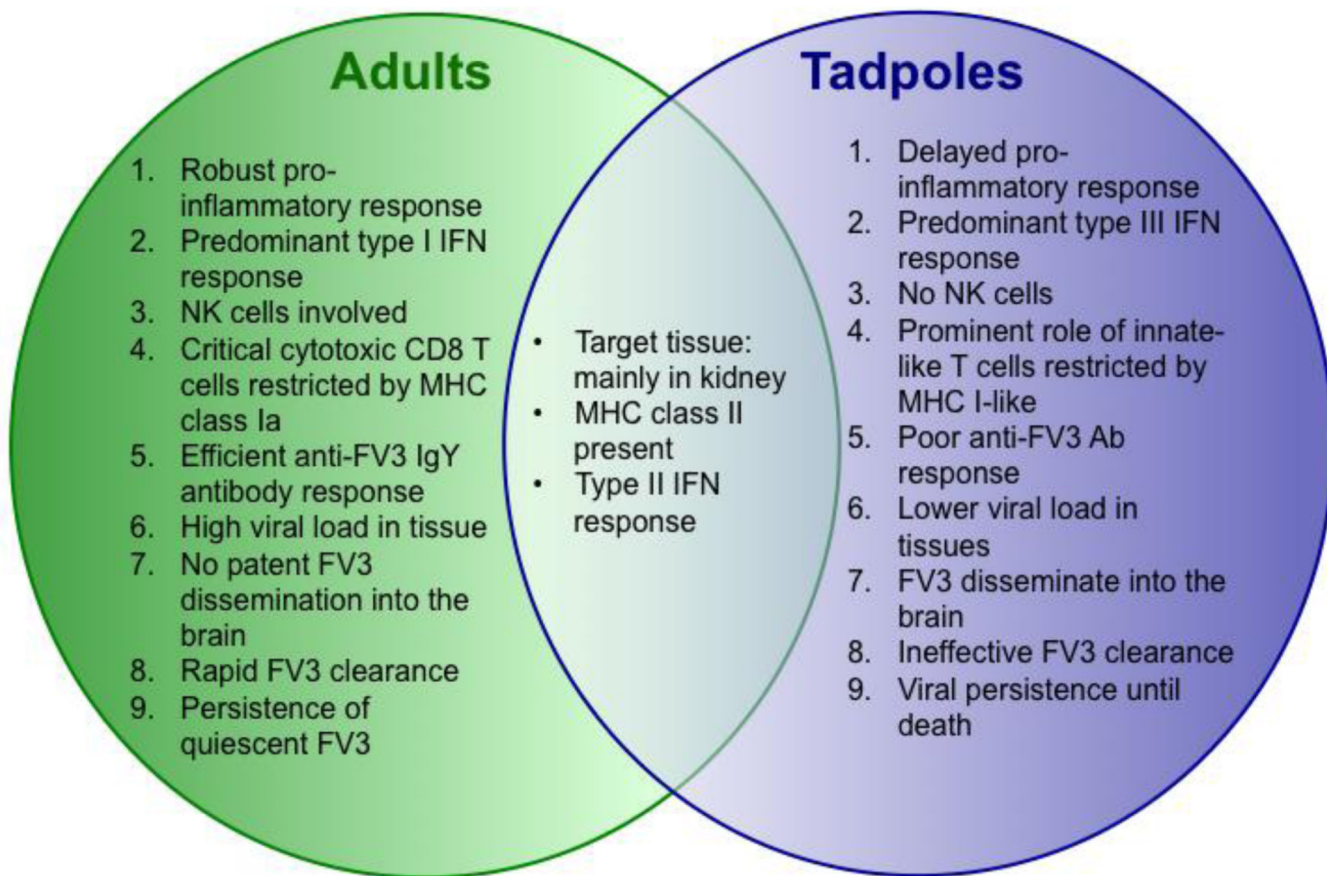


Fig. 1. Overview of the different interactions of FV3 pathogens with the distinct immune systems of *X. laevis* adult and tadpole hosts

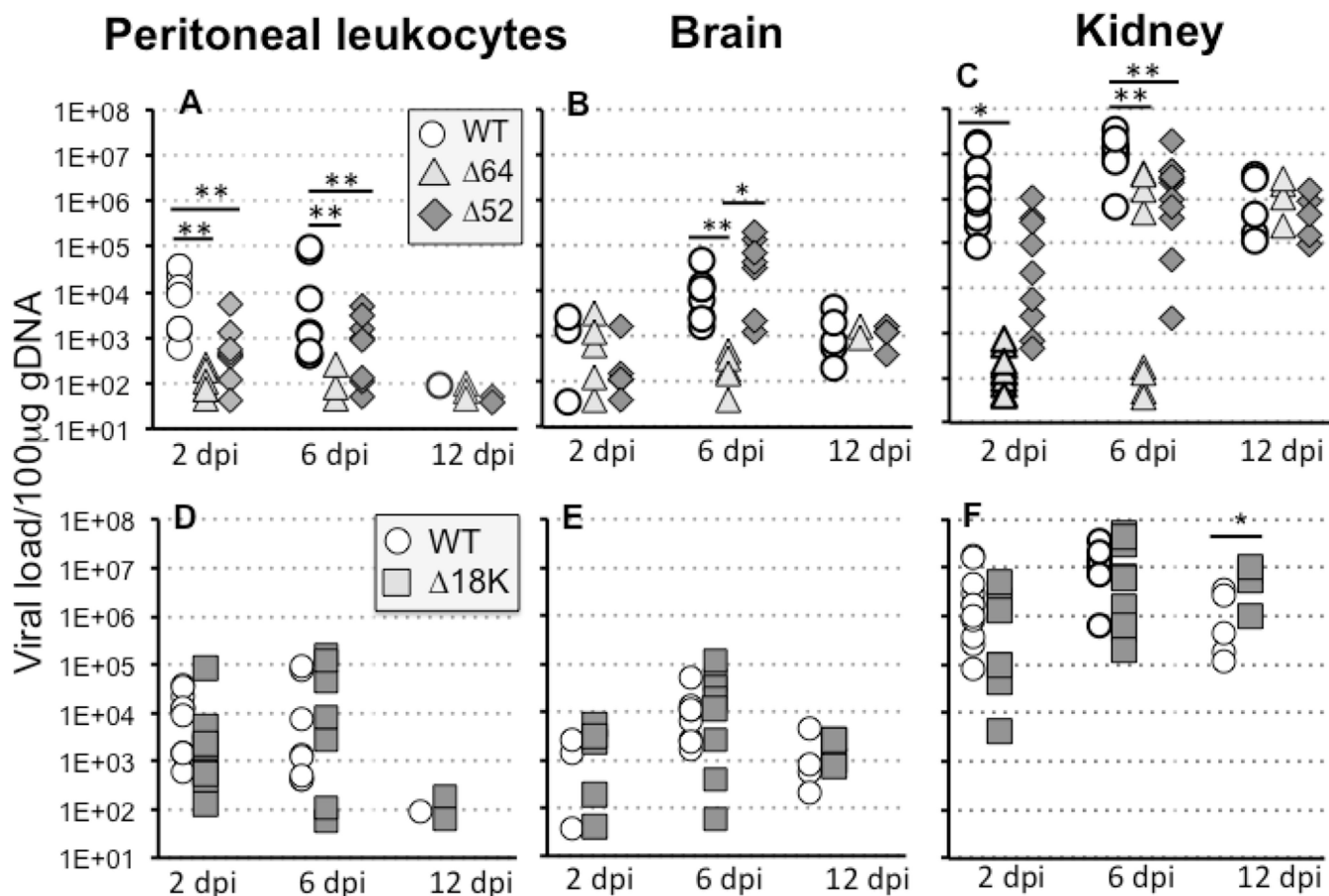


Fig. 2. Viral loads in peritoneal leukocytes (PLs), brain and kidneys of tadpoles at different time following infection with WT-, 52L-, 64R- or 18-FV3

Outbred pre-metamorphic tadpoles were infected by i.p. injection of 1×10^4 PFU of each virus type and FV3 genome copy numbers in PLs, brains and kidneys at 2, 6 and 12 dpi were determined by absolute qPCR using primers specific for FV3 vDNA Pol II. (A–C) Viral loads for 52L-, 64R- and WT-FV3. (D–F) Viral loads for 18K- and the same WT-FV3 control as in A. Results are means \pm SE of genome copy number/100µ of genomic DNA of 6 to 10 animals per group and are representative from two different experiments. **: $P < 0.001$ and *: $P < 0.05$ significant differences between WT and KO-FV3 using one-way ANOVA test and Tukey’s post hoc test.

Tadpole Kidney

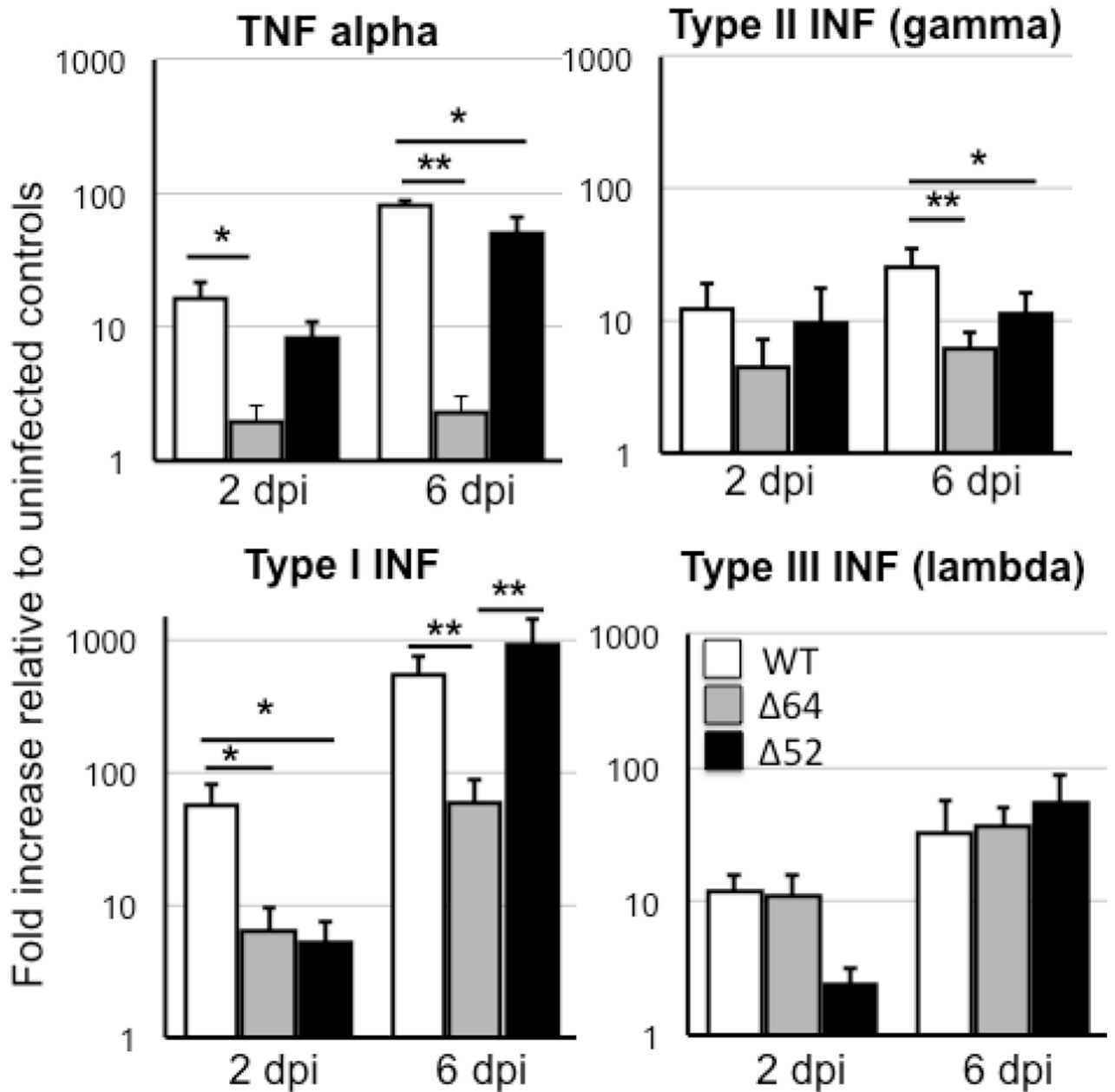


Fig. 3. Changes in expression by RT-qPCR of TNF α , type I, II and III IFN genes in tadpole kidneys during infection with $\Delta 52$ - or $\Delta 64$ -FV3 compared to WT-FV3
 Outbred pre-metamorphic tadpoles were infected by i.p. injection of 1×10^4 PFU of each virus type for 2 and 6 days (dpi). Results are average \pm SEM fold increase relative to uninfected controls of 6 to 10 animals per group and are representative from two different experiments. **, P < 0.001 and *, P < 0.05 significant differences between WT- and KO-FV3 using one-way ANOVA test and Tukey's post hoc test.

Tadpole Brain

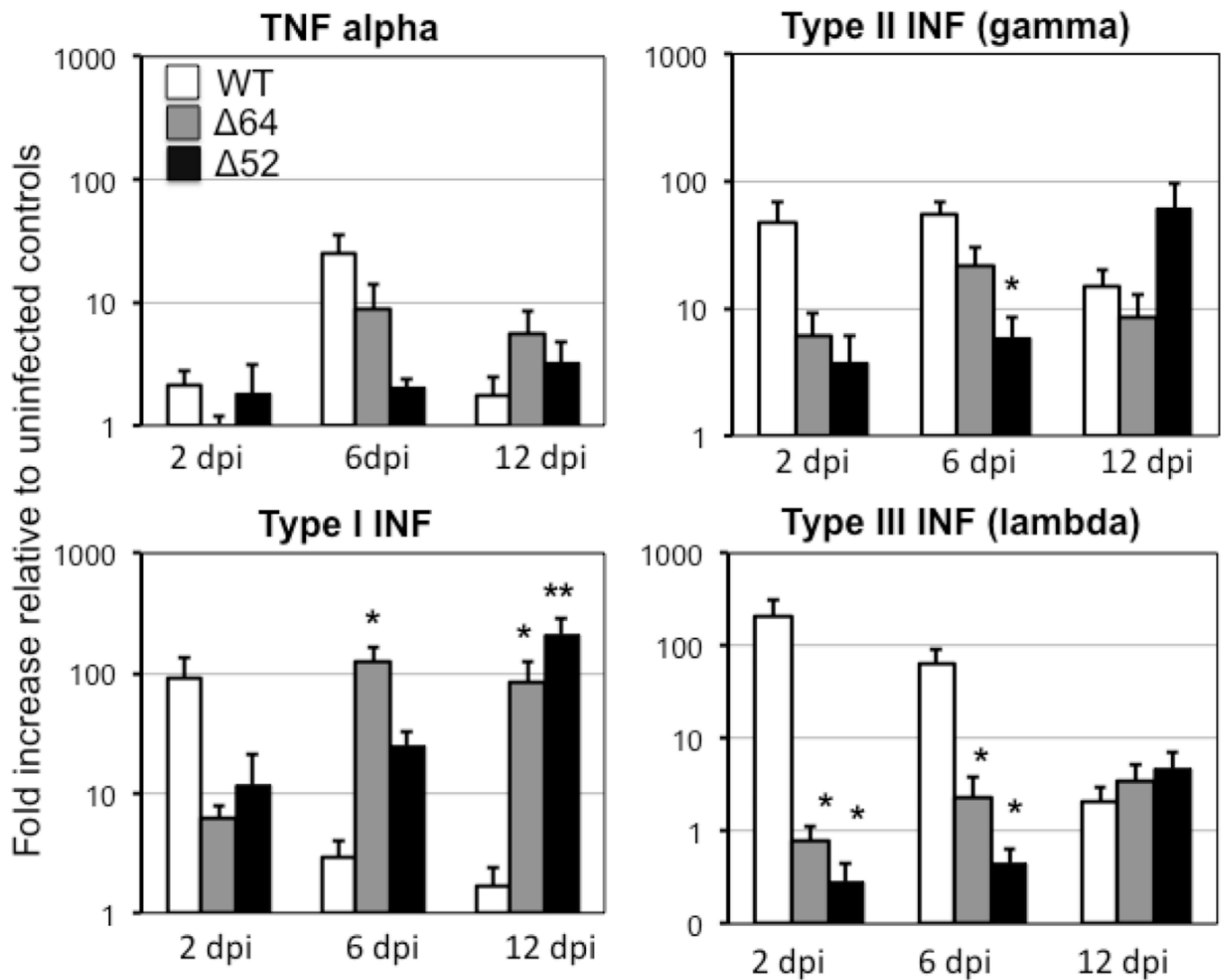


Fig. 4. Changes in expression by RT-qPCR of TNF α , type I, II and III IFN genes in tadpole brains during infection with 52L- or 64R-FV3 compared to WT-FV3
 Outbred pre-metamorphic tadpoles were infected by i.p. injection of 1×10^4 PFU of each virus type for 2, 6 and 12 days (dpi). Results are average \pm SEM fold increase relative to uninfected controls of 6 to 10 animals per group and are representative from two different experiments. **, P < 0.001 and *, P < 0.05 significant differences between WT- and KO-FV3 using one-way ANOVA test and Tukey's post hoc test.

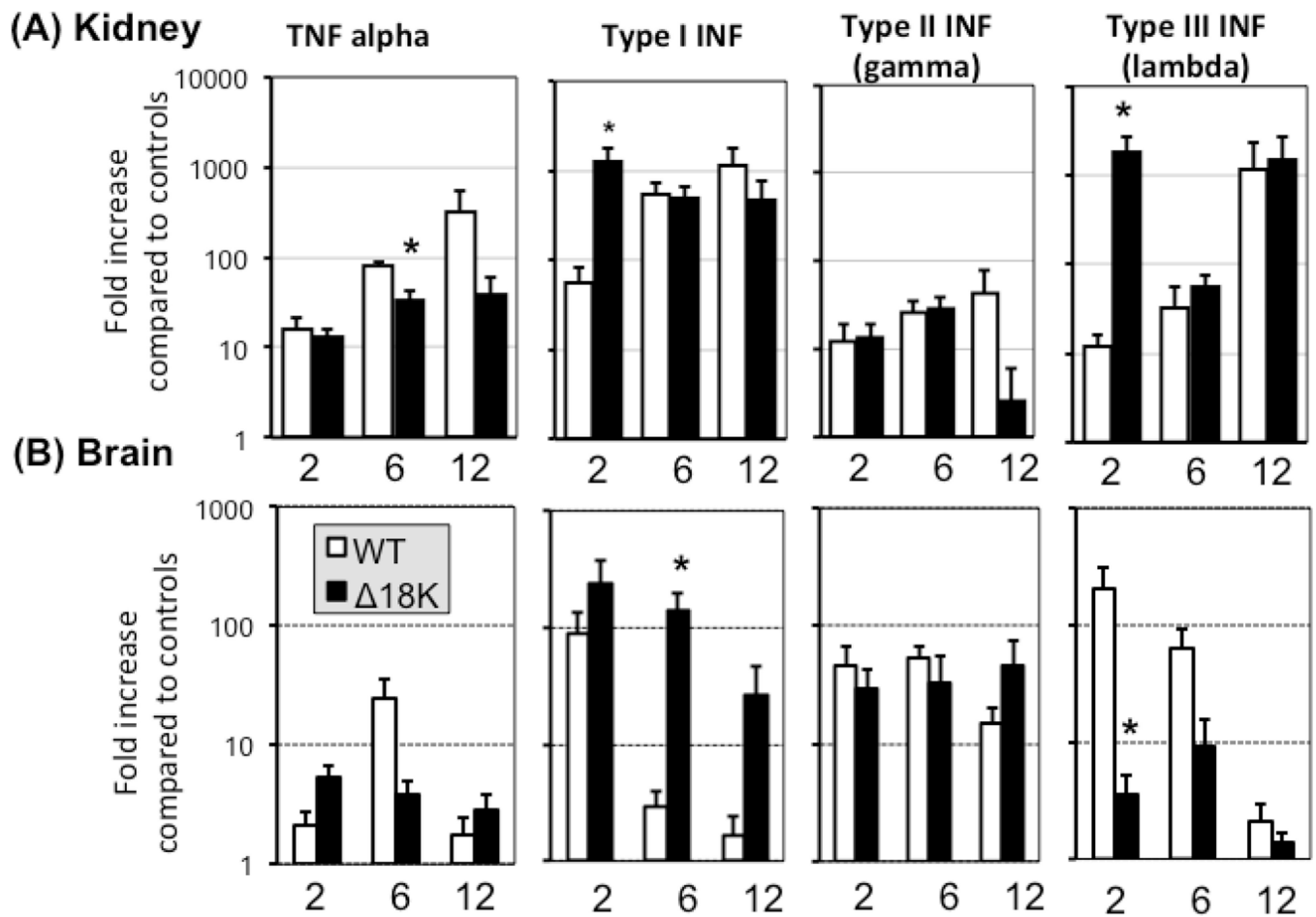


Fig. 5. Changes in expression by RT-qPCR of TNF α , type I, II and III IFN genes in tadpole kidneys and brains during infection with Δ 18K-FV3 compared to WT-FV3
 Outbred pre-metamorphic tadpoles were infected by i.p. injection of 1×10^4 PFU of Δ 18K- or WT-FV3 for 2, 6 and 12 days. Results are average \pm SEM fold increase relative to uninfected controls of 6 to 10 animals per group and are representative from two different experiments. **: P < 0.001 and *: P < 0.05 significant differences between WT- and Δ 18K-FV3 using one-way ANOVA test and Tukey's post hoc test.

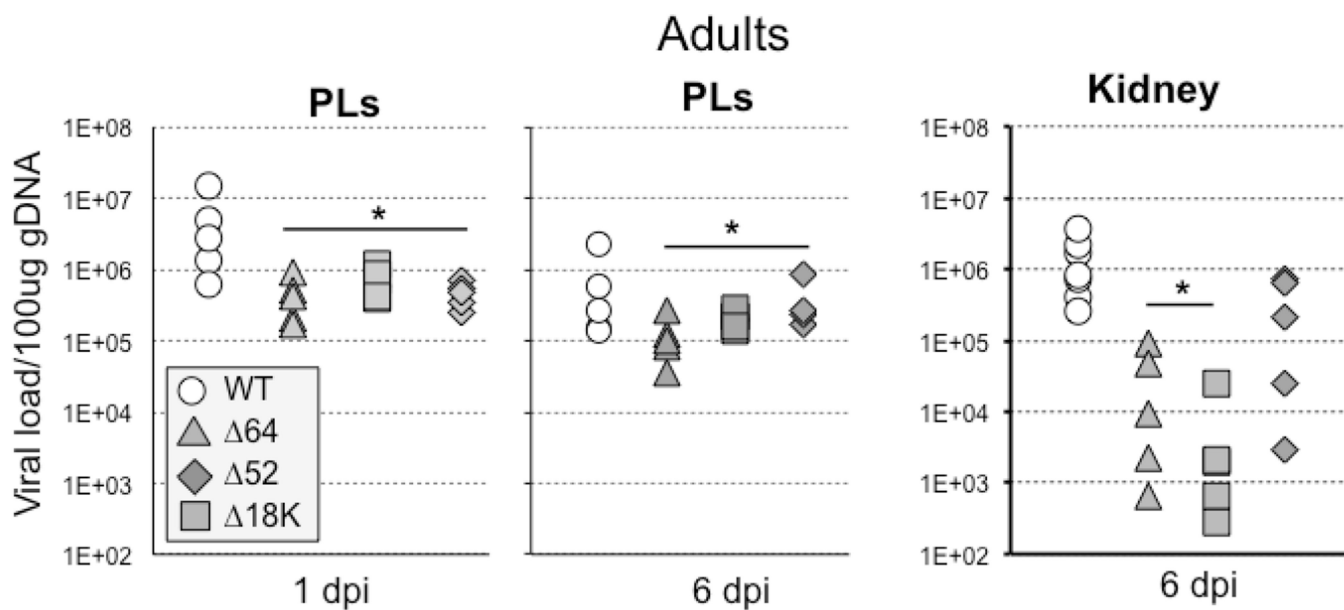


Fig. 6. Viral loads in PLs and kidneys of adult frogs at 1 and 6 days post-infection with WT-, 52L-, 64R- or 18-FV3

Outbred adult frogs were infected by i.p. injection of 1×10^6 PFU of each virus type and FV3 genome copy numbers of PLs and kidneys at 1 and 6 dpi. were determined by absolute qPCR using primers specific for FV3 vDNA Pol II. Results are means \pm SE of genome copy number/100 μ of genomic DNA of 6 to 10 animals per group and are representative from two different experiments. **, P <0.001 and *, P <0.05 significant differences between WT and KO-FV3 using one-way ANOVA test and Tukey as post hoc test.

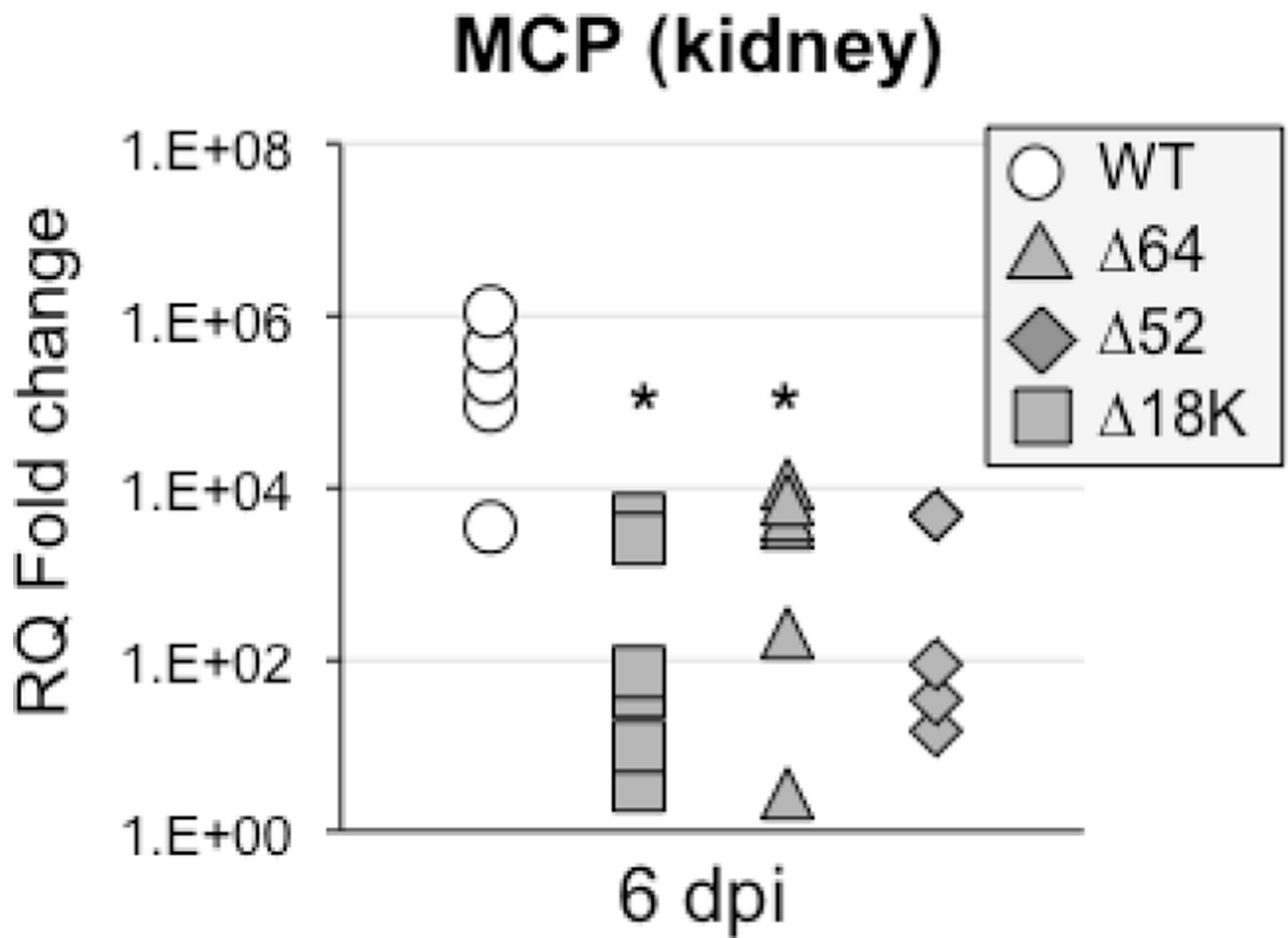


Fig. 7. Changes in expression by RT-qPCR of the viral late gene MCP in infected adult frog kidneys
 Outbred adult frogs (6 individuals per group) were infected by i.p. injection of 1×10^6 PFU of each virus type for 6 days (dpi). Results are average \pm SEM of RQ values. **, $P < 0.001$ and *, $P < 0.05$ significant differences between WT- and $\Delta 18K$ -FV3 using one-way ANOVA test and Tukey's post hoc test

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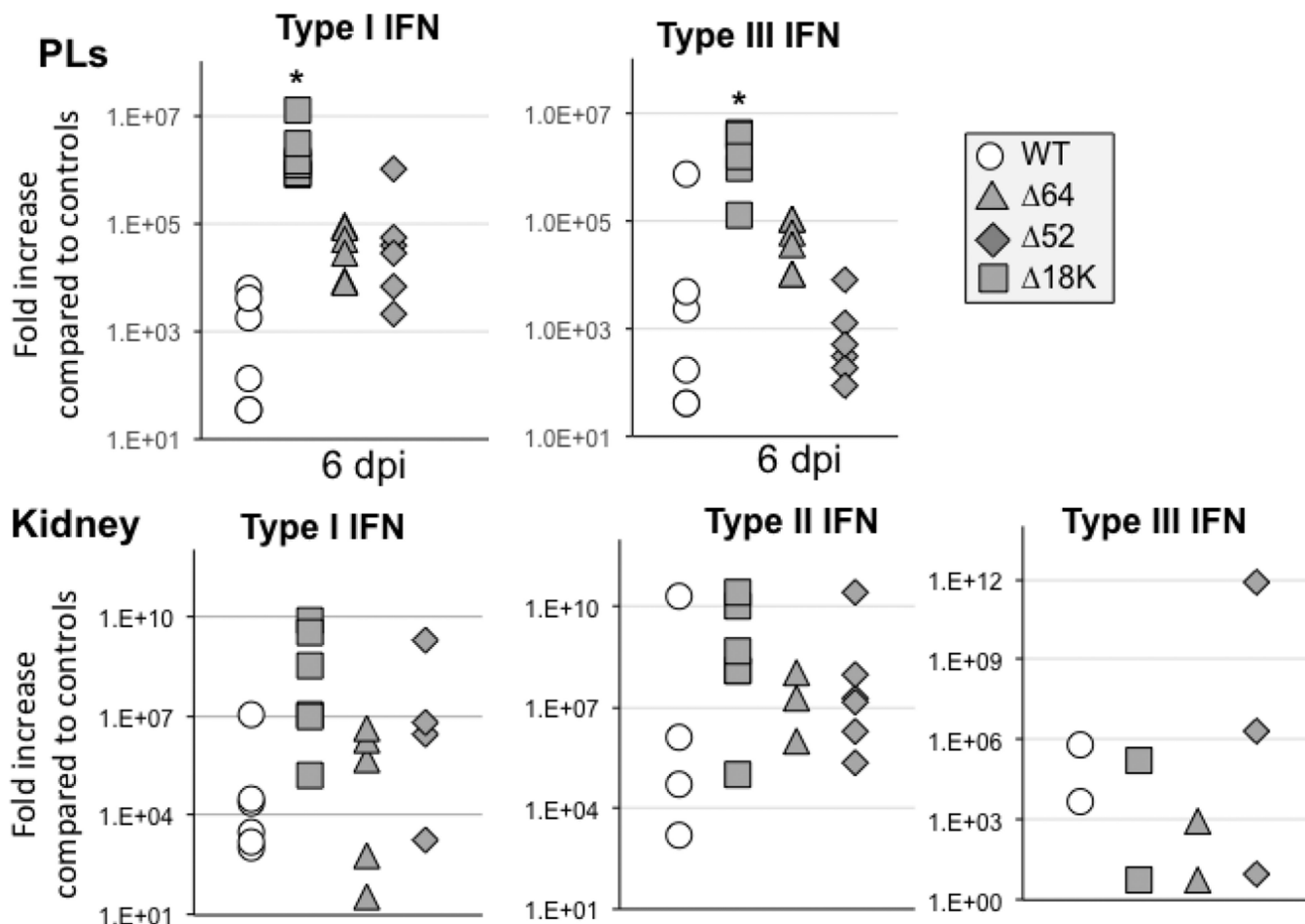


Fig. 8. Changes in expression by RT-qPCR of type I and III IFN genes in adult frog PLs and kidneys during infection with 52L- or 64R- and 18K-FV3 compared to WT-FV3
 Outbred adult frogs (6 individuals per group) were infected by i.p. injection of 1×10^6 PFU of each virus type for 6 days (dpi). Results are average \pm SEM fold increase relative to uninfected controls. **: $P < 0.001$ and *: $P < 0.05$ significant differences between WT- and KO-FV3 using one-way ANOVA test and Tukey's post hoc test.

Table 1

Number of FV3 infectious particles recovered from adult kidney at 6 dpi determined by plaques assay (2 independent experiments using 6 and 3 individual per group, respectively)

Virus	Nb. animals	PFU/ml
FV3-WT	6	1948±90
FV3- 64	6	88±5
FV-3 52	6	144±5
FV3- 18K	6	ND
FV3-WT	3	2440±890
FV3- 64	3	305±91
FV3- 18K	3	70±35

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Table 2

List of primer sequences

PRIMER	SEQUENCE (5'– 3')
DNA Pol II	F: ACGAGCCCGACGAAGACTACA
	R: TGGTGGTCCTCAGCATCC T
GAPDH	F: GACATCAAGGCCGCCATTAAGACT
	R: AGATGGAGGAGTGAGTGTACCAT
Type I IFN	F: GCTGCTCCTGCTCAGTCTCA
	R: GAAAGCCTTCAGGATCTGTGTGT
Type II (gamma)	F:CTGAGGAAATACTTTAACTCCATTGACC
	R:TTGTAACATCTCCACCTGTATTGTC
Type II IFN (lambda)	F: TCCCTCCCAACAGCTCATG
	R: CCGACACACTGAGCGGAAA

F: Forward; R: Reverse. Sequence and more information also available online at <https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis/primers.aspx>